Unravelling the role of glutamine synthetase in seed development in *Arabidopsis thaliana*

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Mestrado em Biologia Funcional e Biotecnologia de Plantas

Departamento de Biologia

2019

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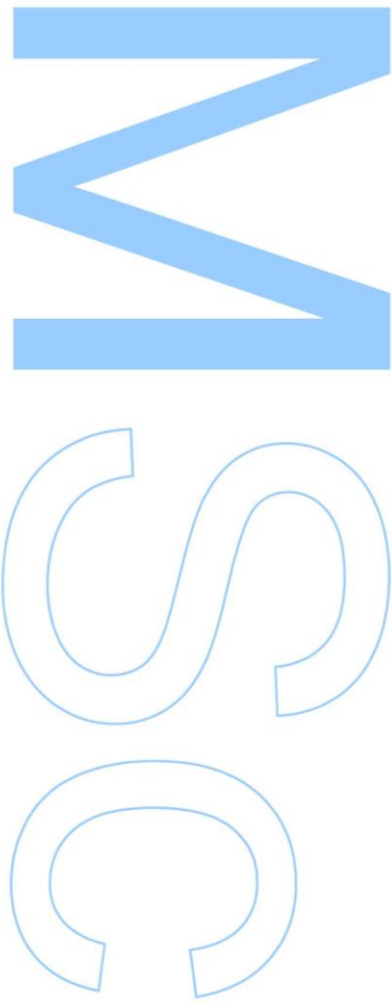

Deus escreve direito por linhas tortas
E a vida não vive em linha recta
Em cada célula do homem estão inscritas
A cor dos olhos e a argúcia do olhar
O desenho dos ossos e o contorno da boca
Por isso te olhas ao espelho:
E no espelho te buscas para te reconhecer
Porém em cada célula desde o início
Foi inscrito o signo veemente da tua liberdade
Pois foste criado e tens de ser real
Por isso não percas nunca teu fervor mais austero
Tua exigência de ti e por entre
Espelhos deformantes e desastres e desvios
Nem um momento só podes perder
A linha musical do encantamento
Que é teu sol tua luz teu alimento

Sophia de Mello Breyner Andresen, Deus Escreve Direito

There is another sky,
Ever serene and fair,
And there is another sunshine,
Though it be darkness there;
Never mind faded forests, Austin,
Never mind silent fields -
Here is a little forest,
Whose leaf is ever green;
Here is a brighter garden,
Where not a frost has been;
In its unfading flowers
I hear the bright bee hum:
Prithee, my brother,
Into my garden come!

Emily Dickinson, There is another sky

Acknowledgements

Tivessem invariavelmente todas as pessoas pelo menos uma oportunidade na vida e talvez tudo tão mais simples. Assim, a minha gratidão para com a Professora Paula Melo e a Professora Sílvia Coimbra, pela oportunidade dada, e mais, pela alternativa também concedida. Acabei por realizar o projecto em que pretendia trabalhar. Obrigado.
À Professora Paula ainda, pela partilha de conhecimento e paciência com que me acompanhou desde o início, ajudando a desenvolver e corrigir este trabalho.
Aos colegas do laboratório SReD pela simpatia e disponibilidade em ajudar.
Obrigado.
Às pessoas do laboratório do Professor Pissarra pela delicadeza com que me receberam, especialmente à Cláudia Pereira, pela pronta ajuda sempre que precisei.
Aos meus amigos, determinantes na construção de carácter. Os que se fazem presentes. Os que o são em bondade, justiça, verdade.
À memória dos meus avós paternos, Ana Conceição Leal da Rocha e Júlio Carlos Alberto Moreira, ao tanto de imaterial que me legaram. A minha eterna gratidão.
À minha mãe, Alda Cristina Barros, a quem tudo devo. Nunca me abandonou.

Resumo

A glutamina sintetase (GS, EC 6.3.1.2) é uma enzima essencial no metabolismo das plantas, catalisando a assimilação de azoto inorgânico, sob a forma de amónio, numa forma orgânica, o aminoácido glutamina. O estudo da GS no crescimento e desenvolvimento das plantas tem despertado especial atenção, tendo como objetivo a melhoria do uso do azoto e o incremento do rendimento das culturas. A enzima existe nas plantas como uma família de isoenzimas, localizadas no citosol (GS1) e nos plastídeos (GS2), apresentando uma multiplicidade de papéis no metabolismo das plantas e estando envolvidas numa ampla variedade de processos fisiológicos, tendo frequentemente papéis não redundantes e não sobreponíveis. A remobilização do azoto nas plantas é essencial para a produção, desenvolvimento e germinação das sementes, portanto o estudo da GS é indispensável para uma melhor compreensão do seu papel nestes órgãos. Em *Arabidopsis thaliana* existem cinco isogenes GS1 sendo dois deles, o *Gln1;5* e *Gln1;3*, os mais expressos nas sementes. O *Gln1;5* é o gene da GS1 com expressão mais elevada e possui uma atividade específica na semente, segundo dados *in silico*. Ambos os genes são alvos pertinentes e não abordados no estudo do desenvolvimento de sementes em *Arabidopsis thaliana*. Assim, este trabalho procura esclarecer a sua importância e, para isso, 1) foram analisados mutantes desprovidos desses mesmos genes (linhas mutantes *gln1;5* e *gln1;3*), o seu fenótipo caracterizado e avaliada a sua importância no desenvolvimento e produção de sementes, bem como nas fases iniciais de germinação; 2) linhas de plantas transgénicas que expressam proteínas repórter foram também criadas e estudadas para ilustrar o padrão de expressão dos genes durante o desenvolvimento das sementes; 3) a expressão dos genes em estudo foi avaliada por RT-qPCR nas plantas selvagens durante o desenvolvimento das sementes e fases iniciais da germinação, e a alteração da expressão dos vários isogenes da GS nos mutantes *gln1;5* e *gln1;3* foram avaliadas, ajudando a definir os papéis fisiológicos destes genes. Nas plantas, várias famílias de genes apresentam redundância funcional, logo a obtenção do duplo mutante *gln1;3*/*gln1;5* foi também um foco deste trabalho.

Os resultados obtidos revelaram apenas pequenas alterações fenotípicas nos mutantes simples, *gln1;3* e *gln1;5*. As plantas *gln1;3* apresentam menor número de hastes florais e síliquas ligeiramente mais curtas, já as plantas *gln1;5* possuíam hastes florais mais longas e um número de folhas da roseta ligeiramente superior. Nenhum dos mutantes simples apresentou diferenças significativas no número de sementes produzido nem no seu fenótipo. A ausência de cada um dos genes não comprometeu a integridade e

viabilidade do pólen ou produziu anomalias no processo reprodutivo. A ausência de fenótipo severo nos mutantes simples levou à criação do mutante duplo *gln1;3*/*gln1;5*, que não foi em tempo útil obtido, não sendo de desconsiderar a sua possível inviabilidade, tendo em conta os resultados de germinação com sementes de mutantes duplo-hemizigóticos. Os ensaios de crescimento de plântulas a diferentes concentrações de azoto mostraram que o gene *Gln1;3* possivelmente terá um papel na remobilização interna do azoto para a radícula durante os primeiros dias de crescimento e, posteriormente, na remobilização de azoto externo para o caule. Os mutantes simples para ambos os genes apresentam menor peso fresco, relativamente à variedade selvagem, na presença de azoto no meio externo, denotando a relevância funcional na absorção de azoto nas fases iniciais do desenvolvimento das plântulas. A expressão destes genes em flores foi confirmada, pela deteção histoquímica de GUS nas linhas repórter, sendo a do *Gln1;5* específica do pólen e a do *Gln1;3* mais ampla. O padrão de expressão durante o desenvolvimento da semente, foi também obtido com sucesso para ambos os genes. Durante a formação da semente o gene *Gln1;3* surge com expressão ao nível dos tegumentos até a semente estar madura, apresentando a partir de então atividade específica ao nível da radícula. O gene *Gln1;5* não apresenta expressão inicial nas sementes com embrião em fase globular e forma de coração, surgindo em embriões já numa fase torpedo mais tardia. Inicialmente a expressão ocorre ao nível dos cotilédones, estendendo-se no decorrer da maturação a todo embrião e semente. Ambos os genes apresentam uma expressão muito limitada à região do hipocótilo 72 horas após a embebição. Os resultados de RT-qPCR revelaram que o *Gln1;3* e *Gln1;5* apresentam expressão crescente durante o desenvolvimento da semente, sendo de facto este último o mais expresso durante o processo. A ausência de *Gln1;3* nas plantas mutantes é principalmente compensada pelo aumento de expressão do gene *Gln1;5*, mas também pelo *Gln1;1,* e a inexistência do *Gln1;5* compensada fundamentalmente pelo gene *Gln1;2*.

Os estudos relativos à importância da glutamina sintetase no desenvolvimento da semente são ainda escassos, principalmente para os genes *Gln1;5* e *Gln1;3*. Desta forma esta dissertação ajuda ao entendimento da eventual importância da GS durante o desenvolvimento da semente, podendo contribuir para a futura melhoria de sementes e desenvolvimento de plantas mais eficientes em termos de utilização do azoto.

Palavras-chave: *Arabidopsis thaliana*, genes *AtGln1;5* e *AtGln1;3*, glutamina sintetase citosólica, desenvolvimento da semente, germinação da semente.

Abstract

Glutamine synthetase (GS, EC 6.3.1.2) is an essential enzyme in plant metabolism, catalyzing the assimilation of inorganic nitrogen, as ammonium, into the amino acid glutamine. GS is considered a bottleneck in plant growth and has received special attention with the goal of improving nitrogen use efficiency and increase crop yield. It exists in plants as a collection of isoenzymes, located in cytosol (GS1) and plastids (GS2), with a multiplicity of roles in plant metabolism and involved in a wide variety of physiological processes throughout plant life cycle, often having non-redundant and non-overlapping roles. Nitrogen remobilization in plants is essential to seed production, development and germination, thus GS study is mandatory to a better comprehension of its role in these organs. *Arabidopsis* has five GS1 isogenes and two of them, *Gln1;5* and *Gln1;3*, are the most expressed in seeds. The *Gln1;5* is the GS1 gene with higher expression and has a seed specific activity, according to *in silico* data. Both genes are pertinent and unstudied targets in *Arabidopsis thaliana* seed development research. This work seeks to clarify the importance of *Gln1;5* and *Gln1;3* genes in seed development, and for that, 1) mutants lacking these genes (*gln1;5* and *gln1;3* mutant lines) were analyzed, phenotype characterized and depicted their importance in seed production and in the first stages of germination; 2) transgenic marker line plants expressing reporter-fusion proteins were created and studied in order to illustrate the genes expression pattern in flowers an during the seed development; 3) the expression of the target genes was evaluated by RT-qPCR in wild-type plants during seed development and in early germination stages. The expression changes of the various GS isogenes, in the *gln1;5* and *gln1;3* mutant plants, was evaluated, helping to define the physiological roles of these genes. As functional redundancy is common in many plant gene families, the double mutant *gln1;3*/*gln1;5* was also a target of this work.

The results of this work revealed that *gln1;3* and *gln1;5* mutant plants had minor phenotypic alterations. The *gln1;3* mutants present fewer floral stems and slightly short siliquae, the *gln1;5* plants have longer floral stems and higher number of rosette leaves. None of the single mutants show significant differences in the number of seeds or their phenotype. The individual lack of these genes does not compromise the pollen integrity, viability, or produced fertilization anomalies. The absence of a severe phenotype in single mutants led to the creation of the *gln1;3*/*gln1;5* double mutant which was not found in a timely manner, being its unviability a possibility, taking into account the double hemizygous mutants germination results. The seedlings growth assays, under different nitrogen concentrations, show that *Gln1;3* possibly has a role in the internal nitrogen

remobilization to the radicles during the first days of growth and later in the uptake of external nitrogen to shoots. The single mutants for both genes present lower fresh weight, than wild-type plants, in external nitrogen presence, denoting their relevance in the first stages of plant development. The expression of these genes in flowers was confirmed by GUS histochemical detection in plants reporter lines, being the *Gln1;5* a pollen specific gene and having the *Gln1;3* a wider expression. During seed development the expression pattern was also successfully characterized for the two genes. Until the seed maturation the *Gln1;3* gene has expression only at seed coat level, showing from mature seeds stage a radicle specificity expression. The *Gln1;5* gene shows no initial expression in seeds with globular and heart shaped embryos, expressing only in later torpedo embryos. Initially the expression occurs at cotyledons level, being then extended to the whole embryo and seed during maturation process. Seventy-two hours after soaking, both genes have their expression restricted to hypocotyl region. The RT-qPCR results revealed that *Gln1;3* and *Gln1;5* have an increased expression during the seed development, being the *Gln1;5* the most expressed GS gene in this organ. The *Gln1;3* absence in mutant plants is mainly compensated by the expression of the *Gln1;5 gene,* and also by the *Gln1;1*. The lack of the *Gln1;5* is mostly compensated by the *Gln1;2* gene.

Glutamine synthetase seed related data is still scarce, particularly on *Gln1;5* and *Gln1;3* genes. Therefore, this research project helps to understand the GS importance during the seed development, possibly contributing to the future improvement of seeds and development of plants with higher nitrogen use efficiency.

Keywords*: Arabidopsis thaliana*, *AtGln1;5* and *AtGln1;3* genes, cytosolic glutamine synthetase, seed development, seed germination.

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List of abbreviations and acronyms

ACR – Act domain repeats
ACT – Actin
ASN – Asparagine synthetase
ATP – Adenosine triphosphate
BP – Border primer
bp – base pair
cDNA – Complementary deoxyribonucleic acid
Col-0 – Columbia 0 variety
CRK – Calcium-dependent kinase-related kinase
CTAB – Cetyltrimethylammonium bromide
DIC – Differential interference contrast
diH_2O – Deionized water
dsDNA – Double-stranded deoxyribonucleic acid
gDNA – genomic deoxyribonucleic acid
DNA – Deoxyribonucleic acid
GDH - Glutamate dehydrogenase
GOGAT – Glutamine oxoglutarate aminotransferase
GS – Glutamine synthetase
GS1 – Glutamine synthetase 1
GS2 – Glutamine synthetase 2
GUS – β-glucuronidase
LB – Luria-Bertani
LP – Left primer
mRNA – Messenger ribonucleic acid
N – Nitrogen
OD – Optical density
PCR – Polymerase Chain Reaction
RCE – RUB1 conjugating enzyme
RNA – Ribonucleic acid
RNase – Ribonuclease
RP – Right primer
RT-PCR – Reverse transcription polymerase chain reaction
RT-qPCR – Reverse transcription polymerase chain reaction quantitative real time
SD – Standard deviation
SDS – Sodium dodecyl sulfate
T-DNA – Transfer DNA
TAE – Tris base, acetic acid and ethylenediaminetetraacetic acid
TAIR – The arabidopsis information resource
Wt – Wild-type

1. Introduction

1.1. Glutamine synthetase: an introductory overview

Climate change and the estimated peak of global population above 11 billion by the end of the 21st century leave us facing two main demands in the near future: feeding this ever-growing population and diminish the agricultural footprint. In order to overcome these problems in a sustainable manner, humankind must pay attention to obtaining higher yields for crops and finding alternatives to the currently highly polluting fertilizers (Lea and Miflin, 2011; Gerland e*t al.*, 2014; Domergue *et al.*, 2019). Nitrogen (N) is the major limiting nutrient in crop production, as it is the one required in largest quantities by plant cells, restraining plant productivity and making the demand of N fertilizers essential in modern agriculture. Although not worldwide available N fertilizers are excessively used nowadays, achieving alarming environmental consequences (Betti *et al.*, 2012; Thomsen *et al.*, 2014). Furthermore, N is still one of the most expensive nutrients to supply, representing a major cost in plant production. These facts lead to the concern regarding the loss of this nutrient in the field, promoting soil and water pollution, and its incomplete capture and poor conversion causing global warming through the emission of nitrogenous oxide. There's also evidence that higher CO_2 values increase the photosynthesis by augmented substrate availability for rubisco (EC 4.1.1.39) and the higher carbon availability often leads to an increase of the C:N ratio, suppressing nitrate assimilation by the plants. So, lowering the N fertilizers input and the improvement of plant N use efficiency is imperative in order to achieve a sustainable agriculture with new N managements strategies (Lea and Miflin, 2011; Betti *et al*., 2012; Thomsen *et al*., 2014; Domergue *et al.*, 2019).

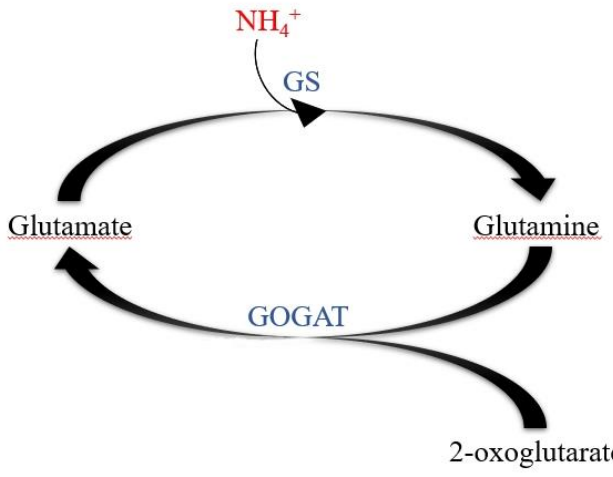


Figure 1 – A simplified view of glutamine synthetase (GS) – glutamate synthetase (GOGAT) cycle.

N is an essential nutrient, constituent of several compounds like nucleic acids and proteins, being determinant for plant growth and development. The inorganic nitrogen absorbed by plant roots, as ammonium (NH_4^+) and nitrate (NO_3^-), has to be assimilated before being processed and becoming part of various organic compounds (Bernard and Habash, 2009; Lea and Miflin, 2011; Fredes *et al.*, 2019). Nitrate, when absorbed, is first reduced in the cytosol into nitrite via nitrate reductase (EC 1.6.1.1), and after that a chloroplast located nitrite reductase (EC 1.7.7.1) will catalyze the nitrite reduction into nitric oxide and ammonium. Finally the ammonium enter in the glutamine synthetase (GS)/glutamate synthetase(GOGAT) cycle (**Figure 1**) being incorporated into low-molecular organic nitrogen compounds, glutamine (Gln) and glutamate (Bernard and Habash, 2009; Lea and Miflin, 2011).

Glutamine synthetase (GS, EC 6.3.1.2) has thus a crucial role in nitrogen metabolism, catalyzing the assimilation of ammonium from different sources to form the amino acid glutamine (Bernard and Habash, 2009; Lea and Miflin, 2011). The first step of GS action in ammonium assimilation is an adenosine triphosphate (ATP) dependent reaction, where the terminal phosphoryl group of ATP goes to the γ-carboxyl group of glutamate to produce the activated intermediate γ-glutamyl phosphate. In a second step an ammonium ion is deprotonated, forming ammonia, this ion binds to the carbonyl carbon to form glutamine and releases one phosphate. Later other enzymes will use this glutamine, the most abundant amino acid in phloem and xylem sap, for the biosynthesis of other nitrogenous compounds (Bernard and Habash, 2009; Lea and Miflin, 2011; Betti *et al.*, 2012). The glutamate used by GS is synthetized by the enzyme glutamate synthase (GOGAT), and its activity is also crucial in nitrogen metabolism. GOGAT acts with GS in a cycle, using the glutamine produced by GS to synthetize glutamate. The reaction is a reductant-driven transfer of the amide amino group of glutamine to 2-oxoglutarate, creating this way two molecules of glutamate (Bernard and Habash, 2009; Lea and Miflin, 2011; Betti *et al.*, 2012).

GS activity is essential for primary ammonium assimilation, but also for its reassimilation and recycling (**Figure 2**) (Oliveira and Coruzzi, 1999; Guo *et al.*, 2004; Betti *et al.*, 2006; Bernard and Habash, 2009; Lea and Miflin, 2011; Thomsen *et al.*, 2014). In plant cells the N assimilation is compartmentalized between the cytosol and chloroplast in relation to different sources of ammonium. Primary sources of ammonium include direct uptake from the soil (at root level) and the reduction of nitrate and atmospheric N_2, secondary sources consist in amino acid catabolism, photorespiratory nitrogen cycling and ammonium produced by phenylalanine ammonia lyase (EC 4.3.1.5) and asparaginase (EC 3.5.1.1). Higher plants have two active forms of GS, with different subcellular

localization and molecular mass: the cytosolic glutamine synthetase (GS1) and the plastid located GS (GS2) (Bernard and Habash, 2009; Lea and Miflin, 2011; Betti *et al.*, 2012; Thomsen *et al*., 2014), being the plastidic GS larger, 44–45 kDa, than the cytosolic one, 38–40 kDa (Bernard and Habash, 2009). Presently it is accepted that GS active isoenzymes are structured as decamers (Unno *et al.*, 2006; Dragićević *et al.*, 2014). These decamers are composed by two face-to-face pentameric rings of identical subunits, with a total of ten active sites, each formed between every two neighboring subunits within each ring. The subunits consist of a smaller N-terminal domain and a larger C-terminal domain with the N-terminal domain of one subunit and the C-terminal domain of the neighboring subunit forming an active site (Unno *et al.*, 2006).

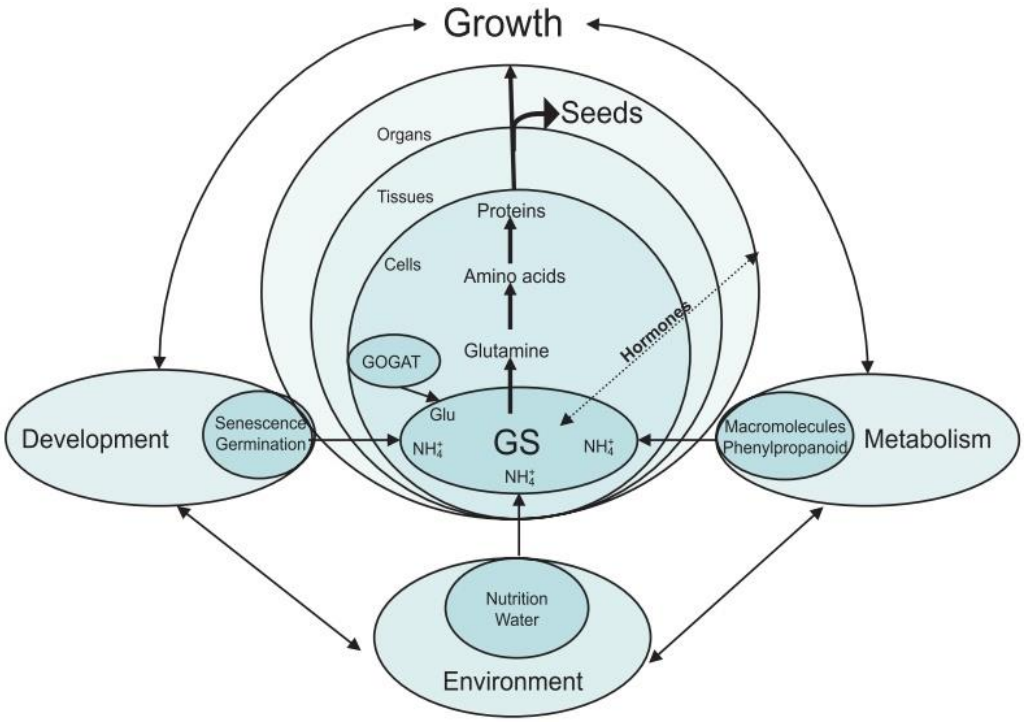


Figure 2 – The central role of glutamine synthetase (GS) in plant growth and seed production. The central circle highlights the function of GS fixing inorganic nitrogen (N) into amino acids that can be transported to developing tissues and organs. Sources of ammonium are highlighted around the central circles. Graphic from Bernard and Habash,2009.

A small family of nuclear genes, with distinct cellular localization and temporal patterns of expression in plants, encode the diversity of GS1 and GS2 isoenzymes, believed to perform non-redundant and non-overlapping roles. The relative abundance of GS isoforms varies between different organs of the same plant and within different plant species, depending on photosynthetic type and/or environmental growth conditions. The GS genes and protein sequences are well conserved both within and across species (Bernard and Habash, 2009; Lea and Miflin, 2011). GS2 is usually encoded by a single gene and GS1 by three to five genes depending on the species (Bernard *et al.*, 2008),

suggesting a more complex role of GS1 in nitrogen assimilation (Bernard and Habash, 2009). Besides the specific details of each GS enzyme form, both assimilate the ammonium produced by diverse plant processes through the same, and equal, reaction (Wang *et al.*, 2013).

The glutamine synthetase isoenzymes are regulated differently, within specific cell types and organs, and in response to different developmental, metabolic and environmental factors. This GS specialization ensures a rapid reassimilation of ammonium derived from multiple sources (Miflin and Habash, 2002). The expression and activity of GS is regulated by a multilevel complex network involving transcriptional, post-transcriptional and post-translational mechanisms, controlled by developmental signals and environmental factors. It has been generally accepted that transcriptional regulation of GS gene family is the main regulatory point controlling GS activity and localization (Lea and Miflin, 2011; Betti *et al.*, 2012). The transcriptional regulation of GS can be mediated by the relative abundance of nitrogen and carbon metabolites. So far, the mechanisms involved in sensing these metabolites levels and linking them to transcriptional regulation are unidentified. Nonetheless, it is known that specific transcription factors directly bind to GS promoter regions or transcripts, regulating the N and C metabolism (Wang *et al*., 2013; Thomsen *et al.*, 2014).

However, growing evidences point to the existence of important post-translational mechanisms regulating GS activity (Bernard and Habash, 2009). Phosphorylation and interaction with 14-3-3 proteins were already observed (Moorhead *et al.,* 1999*;* Lima *et al.*, 2006), nitrosylation (Melo *et al.*, 2011) and oxidation (Ishida *et al.*, 2002) can also interfere with its regulation. The reversible phosphorylation of GS was demonstrated during light/dark transitions (Finnemann and Schjoerring, 2000), and a calcium-dependent kinase-related kinase (CRK3) capable of phosphorylating GS was identified (Li *et al.*, 2006). GS has also been considered a molecular target for nitric oxide being regulated by tyrosine nitration (Melo *et al*., 2011).

The role of GS in plant development, plant nitrogen use efficiency and yield was highlighted in several studies, particularly in cereals, like maize and rice (Habash *et al.*, 2001; Tobin and Yamaya, 2001; Limami *et al.*, 2002; Miflin and Habash, 2002; Tabuchi *et al.,* 2005; Bao *et al.*, 2014). The GS relevance in ammonium assimilation is fully asserted, and also stated the inability of glutamate dehydrogenase to fulfill the GS role. So the ongoing study of GS genes, their individual roles and interactions with other enzymes related to nitrogen metabolism has potential for the agronomic future (Lea and Miflin, 2011; Orsel *et al.*, 2014). The understanding of GS regulation mechanisms is critical to meliorate plant performance and increase crop productivity. In that way, several

studies devoted to this enzyme were already performed with the goal of improving N use efficiency and crop yield (Thomsen *et al.*, 2014).

1.2. Cytosolic Glutamine synthetase (GS1): several genes to fulfill the same function?

Cytosolic glutamine synthetase is the major form of GS active in plant roots and the ammonium taken up from the soil is directly converted into glutamine by its action. In order to alleviate high levels of ammonium toxicity the plants reduce its transport to the shoots increasing ammonium assimilation by the roots whereby GS1 has a determinant role (Tingeys *et al*., 1988; Peterman and Goodman, 1991; Guan *et al*., 2016; Moison *et al*., 2018). This was demonstrated in cereal species like rice, which has three GS1 isogenes, with *OsGS1;1* gene being critical in plant growth and *OsGS1;2* the gene determinant for primary root ammonium assimilation (Funayama *et al.*, 2013). The importance of GS1 activity in phloem and related vascular tissues was also confirmed. As glutamine is one of the major forms of amino acid participating in N remobilization through phloem. Later it ends up being converted into glutamate, delivering nitrogen in the collector tissues (Tobin and Yamaya, 2001; Lea and Miflin, 2011; Moison *et al.*, 2018). A GS1 senescence related activity is also described (Guo *et al.*, 2004; Avilla-Ospina *et al.*, 2015; Moison *et al.*, 2018). During leaf senescence, ammonium is released via amino acid catabolism, the chloroplasts and consequently the plastidic glutamine synthetase (GS2) are degraded, and a compensation mechanism where some GS1 isoforms are induced is activated *(Guo et al., 2004; Diaz et al.*, 2008; Orsel *et al.*, 2014; Avilla-Ospina *et al.*, 2015; Ji *et al.*, 2019).

As GS1 gene family in plants evolved by duplication of an ancestral cytosolic GS gene and the evolution occurred to fulfil different metabolic requirements (Bernard and Habash, 2009), different GS1 isogenes are likely to have specific and non-redundant roles in the N metabolism. In rice this non-redundant role of GS1 isogenes is clear, the genes *OsGS1;2* and *OsGS1;3* can't compensate the lack of *OsGS1;1*, and this last can't compensate the *OsGS1;2* absence (Tabuchi *et al.,* 2005; Funayama *et al.*, 2013). In maize (*Zea mays*), which has five GS1 isogenes, was asserted that *ZmGln1;3* and *ZmGln1;4* have non-redundant roles as well (Martin *et al.*, 2006), and different expression patterns observed in species like *Brassica napus* suggest that all the sixteen different GS1 isogenes have non-redundant activity (Orsel *et al.*, 2014). Although these evidences on GS1 isoenzymes non-redundancy, it is also described in literature that

GS1 isoenzymes can functionally complement each other in *A. thaliana* mutants (Dragićević *et al.*, 2014; Guan *et al.*, 2015; Konishi *et al.*, 2017; Moison *et al.*, 2018).

In maize plants GS1 has low levels of expression in young photosynthetically active leaves, however its content and activity increase as the plant grows older, when nitrogen remobilization from mature leaves and grain filling are occurring (Habash *et al.*, 2001). The *ZmGln1;1* maize gene has major expression in roots, and the *ZmGln1;2* is preferably expressed in the developing kernel, pedicel, pericarp and also present in the anthers, glumes and ear husks (Lea and Miflin, 2011). The *ZmGln1;3* isogene expresses constitutively in mesophyll cells, suggesting that this GS1 isogene has activity related with ammonium assimilation from the nitrate reduction (Martin *et al.*, 2006), the *ZmGln1;4* is more expressed in older leaves and *ZmGln1;5* seems to have generalized low expression (Hirel *et al.*, 2005; Lea and Miflin, 2011).

In rice it was verified that cytosolic GS expression has vascular bundle specificity, also highlighting its role on nitrogen remobilization, eventually from older leaves to new ones (Tobin and Yamaya, 2001). The lack of *OsGS1;2* isogene in rice causes a marked decrease of nitrogen content in shoots at vegetative and reproductive stages (Funayama *et al.,* 2013). The absence of *OsGS1;1* gene, which expression is higher in roots, produces severe retardation in plant growth and *OsGS1;3* isogene seems to be a spikelet specific GS1 gene in rice (Tabuchi *et al.*, 2005). Overall, in cereal plants the overexpression of GS1 isogenes seem to produce improvements on nitrogen metabolism. Nonetheless, this changes usually are not significant, possibly due to still uncharted mechanisms in the GS1 regulation that affect the full potential benefit of GS1 overexpression (Bao *et al.*, 2014; Thomsen *et al.*, 2014).

1.3. Plastidic glutamine synthetase (GS2): a photorespiratory enzyme

The dominating role of plastidic glutamine synthetase is in the reassimilation of photorespiratory ammonium at chloroplast level, and its assimilation deriving from the nitrite reduction in plastids. The GS2 has major expression at green tissues level, being the most expressed form in green leaves (Wallsgrove *et al.*, 1987; Orea *et al.*, 2002; Thomsen *et al.*, 2014). GS2 is particularly regulated in order to coordinate inorganic nitrogen assimilation with the available carbon backbones photosynthetically produced, kidnapping the ammonia synthetized during photorespiration (Oliveira and Coruzzi, 1999). Even though its presence was already reported also on non-photosynthetic tissues (Woodall and Forde, 1996; Orea *et al.*, 2002; Melo *et al.*, 2003) and absent in the

conifer family (Lea and Miflin, 2011). The GS2 is encoded by one unique gene in most plants, though, it was already recognized a second plastidic GS gene in *Medicago truncatula* expressed only in developing seeds (Seabra *et al.*, 2010).

The primary amino acidic sequences of cytosolic and plastidic GS are very similar, differing by additional N- and C- terminal extensions in plastidic GS proteins, around 60 and 16 more residues, respectively (Lightfoot *et al.*, 1988; Melo *et al.*, 2003). Plastidic GS is a nuclear encoded protein initially synthesized in cytosol as a higher molecular mass precursor polypeptide containing a cleavable N-terminal extension, the transit peptide (Lightfoot *et al.*, 1988; Tingeys *et al.*, 1988). This N-terminal will then direct the polypeptide into the chloroplast, where it is cleaved by stromal processing peptidases. Inside the organelle, GS2 polypeptides will assemble to form the catalytically active enzyme (Keegstra and Cline, 1999; May and Soll, 1999). Recently was determined in *Medicago truncatula* that GS2 C-terminal extension peptide was relevant for the enzyme activity. This C-terminal promoted the interaction between GS and glutamate, although it was not determinant for the chloroplast enzyme import process (Ferreira *et al.*, 2017).

Photorespiratory mutants were first described in 1979 (Somerville and Ogren, 1979) and, since then different types of photorespiratory mutants were isolated (Betti *et al.*, 2012), and described as capable of normal growth under non-photorespiratory conditions being non-viable under photorespiratory conditions (Blackwell *et al.*, 1987; Wallsgrove *et al.*, 1987). However, photorespiration mutants lacking GS2 were only isolated in barley and *Lotus japonicus*, confirming the role of GS2 in the assimilation of photorespiratory ammonium. A *Lotus japonicus* mutant revealed that GS2 has also an important role in the plant nodules and in legumes productivity (Betti *et al.*, 2012). Studies also showed that GS2 activity in the leaves declines in response to drought or salt stress, whereas GS1 tends to increase or maintain the same level of activity (Bauer *et al.*, 1997; Lutts *et al.*, 1999; Martinelli *et al.*, 2007). But the role of GS2 in drought or salt stress is controversial, as some studies highlight GS2 as a key player in plant tolerance to salt stress (Hoshida *et al*., 2000) or drought (Díaz *et al.*, 2010) while some studies assert an enhancement of the stress resistance mechanisms in the GS2 absence (Pérez-delgado *et al*., 2013; Ferreira *et al*., 2019).

The GS2 plays a role in crop productivity (Ferreira *et al.*, 2019), and it is been also suggested that low GS2 activity levels may limit leaf function during the late grain filling stage (Hu *et al.*, 2018), confirming that GS2 is involved in plant N use efficiency and biomass production interacting with GS1 to balance nitrogen and carbon metabolism of the plants as a whole. (Bao *et al.*, 2014; Németh *et al.*, 2018).

1.4. Glutamine synthetase in the model plant *Arabidopsis thaliana*

In *Arabidopsis thaliana* the GS gene family has five genes coding for different GS1 isoenzymes, *Gln1;1* to *Gln1;5* (The *Arabidopsis* Genome Iniciative, 2000; Ishiyama *et al.*, 2004), which were studied by using knockout mutants (Lothier *et al.*, 2011; Dragićević *et al.*, 2014; Guan *et al.*, 2015; Guan *et al.*, 2016; Konishi *et al.*, 2017; Konishi *et al.*, 2018; Moison *et al.*, 2018; Ji *et al.*, 2019), encoding for proteins with sequence homology between 79 and 92%, and one GS2 gene, *Gln2*. The GS1 gene products can assembly into active heterodecameric enzymes (Dragićević *et al.*, 2014).

The GS1 isogenes, and their individual roles, are fairly well studied and its expression, mainly in vascular tissues, has been ascertained in *A. thaliana* (**Figure 3**), synthetizing glutamine that is essential to ammonium primary assimilation in roots, remobilizing nitrogen during leaf senescence and involved in the maintenance of redox homeostasis in chloroplasts underlying stress responses, modulating cellular ROS (Bernard and Habash, 2009; Betti *et al.*, 2012; Moison *et al.*, 2018; Ji *et al.*, 2019).

The *Gln1;1* gene has expression in epidermal root cells, in the elongation zone, corroborating that it possibly has a role in sensing and on the assimilation of exogenous nitrogen. The *Gln1;1* correspondent isoenzyme has high ammonium affinity, participating on nitrogen transport from the exterior to the plant (Ishiyama *et al.*, 2004) and being also involved and playing non-redundant roles in stress responses, indispensable in *Arabidopsis* tolerance on various abiotic stresses (Dragićević *et al.*, 2014; Ji *et al.*, 2019). The shorter roots of *gln1;1* single mutants suggest, like previously referred by Ishiyama *et al.* (2004), that this isogene expression is part of a signaling pathway mediating modifications in the root system as a response to nitrogen supply (Guan *et al.*, 2015; Guan and Schjoerring, 2016). Apparently under standard nitrate conditions the *gln1;1* single and double mutants don't present severe phenotype (Moison *et al.*, 2018) and is described in *gln1;2* and *gln1;3* single mutants the *Gln1;1* gene induction to compensate the lack of *Gln1;2* and *Gln1;3* transcripts (Dragićević *et al*., 2014).

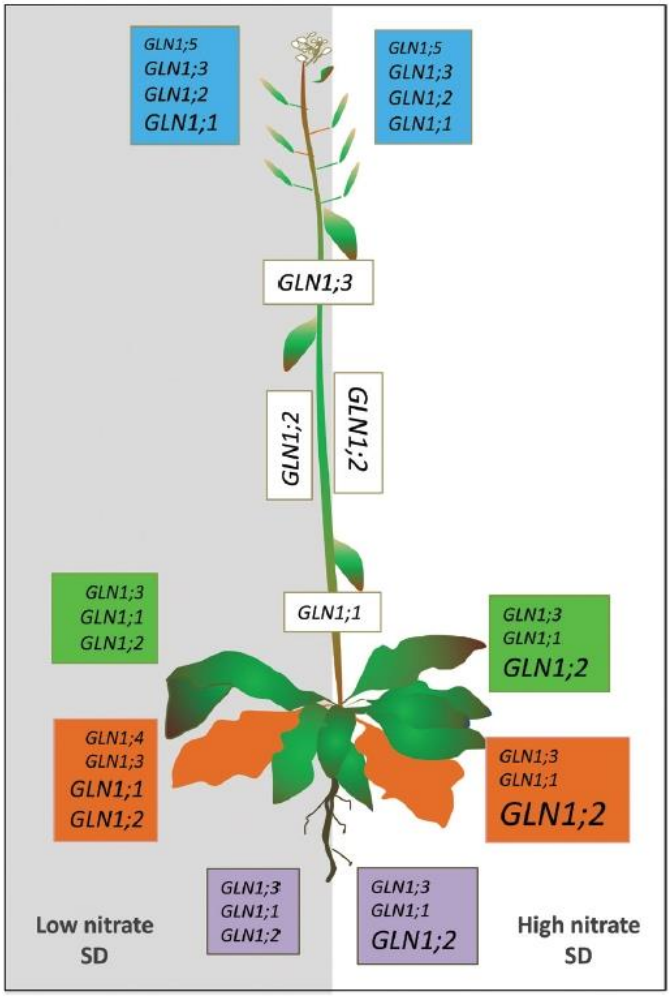


Figure 3 – Schematic representation of *Gln1* isogenes expression in flowers (blue), stem (white), young leaves (green), old leaves (orange) and roots (violet) of *Arabidopsis thaliana* grown under short days (SD) and either low (left, grey background) or high (right, white background) nitrate conditions. Relative expression levels are represented by the relative size of the gene names. Graphic image from Moison *et al.* (2018).

The *Gln1;2* codes for an isoenzyme with low ammonium affinity, essential for plant vegetative growth and ammonium assimilation, being the GS gene with higher expression levels in leaves. Its transcripts are abundant in all plant tissues except in mature pollen and seeds (Schmid *et al.*, 2005; Lothier *et al.*, 2011; Guan *et al.*, 2016). Under standard N concentrations the *gln1;2* mutants are smaller than wild-type plants, and ammonium toxicity symptoms are more evident in these mutants, having also decreased glutamine content. While in *gln1;1* single mutant the first toxicity symptoms appear on the younger leaves, in the *gln1;2* plants these symptoms appear also on mid-to-older leaves (Guan *et al.*, 2015; Guan *et al.*, 2016). GLN1;2 is the main isoenzyme that contributes to GS1 activity in shoots at vegetative growth stage. To relieve ammonium toxicity the *Gln1;2* gene can be upregulated, having a role in germination and rosette biomass, and its expression in shoots is upregulated by high ammonium supply

(Guan *et al.*, 2015; Guan *et al.*, 2016; Ji *et al.*, 2019). The double mutant *gln1;1*/*gln1;2* has glutamine synthesis compromised, resulting in ammonium accumulation and a severity of phenotype higher than in *gln1;2* single mutants. The high abundance of GS1 protein in these single and double mutants despite of the lack of upregulation of other GS1 isogenes suggests post-transcriptional regulation of the GS1 transcripts in high ammonium availability (Guan *et al.*, 2016).

The pericycle is one of the locations where *Gln1;3* gene has expression, so this isogene can possibly be associated with xylem loading of glutamine in plants, and in *Arabidopsis* it only reveals its function of ammonium assimilation in the roots when the *Gln1;2* isn't functional (Konishi *et al.*, 2017). Like *Gln1;2* gene, the *Gln1;3* encodes an isoenzyme with low ammonium affinity and has the highest capacity for glutamine synthesis, being also essential in roots for N metabolism (Ishiyama *et al.*, 2004; Moison *et al.*, 2018). Under external ammonium treatments of different concentrations, the *Gln1;3* expression seems to maintain unchanged in both shoots and roots. Its main expression is related to flowers, buds, seeds and seems to occur, specifically, in plant low-order veins (Guan *et al.*, 2016; Moison *et al.*, 2018). The loss of function of *Gln1;1* and *Gln1;3* genes leads to poor germination and the triple mutant *gln1;1*/*gln1;2*/*gln1;3* is more severely affected, having the leaves of this triple mutant greater total amino acid and ammonium contents than wild-type plants (Ji *et al*., 2019). The *gln1;2* and *gln1;3* single mutants don't show phenotype under low ammonium conditions, and with high NH_4^+ supply only the *gln1;2* plants showed biomass reduction (Konishi *et al*., 2018). It is estimated that the GLN1;3 isoform together with the GLN1;1 and GLN1;2, contribute with 10-20% of the total N remobilization in *Arabidopsis thaliana,* having supposedly the three isoenzymes redundant roles only at lower ammonium conditions (Konishi *et al*., 2018; Moison *et al*., 2018). The GS1 structural analysis reveals that different GS subunits have combinatory potential. The subunits affinity and plasticity in assembly active holoenzymes suggested that GLN1;1 and GLN1;2 normally bind to GLN1;3, but in GLN1;3 absence the other two combine easily with each other (Dragićević *et al.*, 2014).

The *Gln1;4* isogene, like *Gln1;1*, encodes an isoenzyme with high affinity for ammonium but further research is needed to understand their ammonium assimilatory mechanisms (Konishi *et al.*, 2017; Moison *et al.*, 2018). The *Gln1;4* induction in leaf senescence, like *Gln1;1* and *Gln1;2*, has already been amply described (Bernhard and Matile, 1994; Guo *et al.*, 2004; Ishiyama *et al.*, 2004; Diaz *et al.*, 2008; Lothier *et al.*, 2011; Avilla-Ospina *et al.*, 2015;), and it seems that *Gln1;4* might participate in ammonia reassimilation, released by photorespiration (Wang *et al.*, 2013). As the *Gln1;1* gene, the *Gln1;4* is preferentially expressed in low-nitrogen conditions, having higher expression in roots.

The lack of these two genes, at low N levels can be compensated by the other GS1 isoenzymes. As *Gln1;1* and *Gln1;2* genes, the *Gln1;4* seems to be preferably expressed in high-order veins (Moison *et al.*, 2018), shoots and roots (specially at pericycle cells in the basal region of lateral roots emergence), and after ammonium treatments its expressions levels remain, still, very low (Ishiyama *et al.*, 2004; Guan *et al.*, 2016). The double mutant *gln1;2/gln1;4* seems to have a lethal effect on plants and the *gln1;1/gln1;4* was already studied and does not show a reduction in biomass under ammonium limited conditions (Konishi *et al.*, 2017; Konishi *et al.*, 2018). Towards the absence of *Gln1;1* and *Gln1;3,* the *Gln1;4* gene seems to be essential in male fertility (Ji *et al.*, 2019).

The expression of *Gln1;5* gene, as the *Gln1;3,* is specific of the low-order veins and is absent from roots and rosettes being its expression seed and pollen specific, although there is no data available about its possible roles (Ishiyama *et al.*, 2004; Lothier *et al.*, 2011; Moison *et al.*, 2018). Vegetative tissue samples of a *Gln1;5* knockout plant were analyzed by RT-qPCR, and showed to be similar to wild-type, confirming the specific expression in reproductive tissues (Dragićević *et al.*, 2014; Moison *et al.*, 2018). Of the five isogenes, *Gln1;5* and *Gln1;3* are the two mainly expressed at seed and pollen level (Lothier *et al.*, 2011; Moison *et al.*, 2018). As already stated, the comparison of *Arabidopsis* cytosolic GS isoforms patterns has indicated that these isoenzymes can combine between them in all proportions forming heteromeric proteins, however no data is available about GLN1;4 and GLN1;5 possible combinations between them and with the remaining GS isoenzymes (Dragićević *et al.*, 2014).

The GS2 gene in *A. thaliana*, *Gln2*, was recently studied using *gln2* single mutants. The mutant plants showed an impairment in the ammonium assimilation, displaying a dwarf phenotype under photorespiratory conditions, although being viable, suggesting a redundancy of activity with cytosolic GS and GDH in photorespiratory ammonium assimilation (Ferreira *et al.*, 2019). The *Gln2* absence is compensated by an increase in cytosolic GS genes, particularly the *Gln1;2* and *Gln1;3 and by* glutamate dehydrogenase (GDH) whose activity and expression is enhanced and might account for the increased tolerance to salt stress exhibited by these mutants (Ferreira *et al.*, 2019). Whereas the study of the *gln1;1/gln1;2/gln1;3* triple mutant in *Arabidopsis* showed that the absence of these GS1 genes promotes higher values of *Gln2* and protein content, indicating that GS2 can also, in some way, compensate the lack of GS1, although not having the capability *per se* to restore the wild-type phenotype in *Arabidopsis* (Moison *et al.*, 2018), and further, confirming the redundancy of functions between GS isoenzymes present in *A. thaliana*. The GS2 presence in mitochondria of *Arabidopsis thaliana* plants was described (Taira *et al.*, 2004), although no more data related emerged. The *Gln2* gene

is associated with a uridylyltransferase-like protein, called Act Domain Repeats 11 (ACR11), that acts as an activator, and responsible for the GS2 mechanistic role in nitrogen assimilation (Osanai *et al.*, 2017). GS2 holoenzymes can dissociate into lower molecular weight multimers with higher electrophoretic mobility (Mäck and Tischner, 1994; Betti *et al.*, 2006; Dragićević *et al.*, 2013) and this particularity can explain the absence of GS2 among decameric isoforms studied so far, not combining with GS1 protein subunits (Dragićević *et al.*, 2014).

Besides all this information the physiological functions of GS genes in *Arabidopsis*, that are differently expressed depending on the plant organ, the photoperiod, and the nitrate conditions, have been only partially studied. The existent researches lack of seed GS data, as the central attention go to nitrogen remobilization in aerial organs and the predominant expressions in vascular tissues. (Thomsen *et al.*, 2014; Guan *et al.*, 2015; Guan *et al.*, 2016; Konishi *et al.*, 2017; Konishi *et al.*, 2018; Moison *et al.*, 2018; Ji *et al.*, 2019). The similarities found so far between the GS enzymes are encouraging to study and transfer the knowledge from *A. thaliana* model plant to *B. napus* or even to other more distanced species with commercial interest, like maize or wheat (Lea and Miflin, 2011; Orsel *et al.*, 2014).

1.5. Seed development and germination: does GS take a role?

The improvement of seed quality and productivity is amongst the most crucial approaches to meet the food requirements for the present ever-growing population (Gerland *et al.*, 2014) . Seeds are the major providers of our caloric intake, directly as food or indirectly through animals feed and represent a market of 30 billion euros. Food and Agriculture Organization stands that a seed germination rate equal to 80% in cereals and 60-80% in other species is acceptable in terms of seed quality standards, thus still existing a considerable potential for seed quality improvement (Domergue *et al.*, 2019). Therefore, intense efforts are being devoted to improve seed quality, mostly using genomic tools. Furthermore, understanding the fundamental processes taking place in seeds during their development, storage, germination and growth is imperative to increase the productivity of crop species, exploiting their potential in plant breeding (Gerland *et al.*, 2014; Wang *et al*., 2015; Domergue *et al.*, 2019).

The seed development starts when two male gametes fuse with their female counterparts, originating the diploid embryo and the triploid endosperm (Figueiredo and Köhler, 2018). The development comprises two distinct phases, the embryonic development and the seed maturation phase. The embryogenesis, a phase of morphogenesis, begins with the formation of a single cell zygote and ends at the heart

shape stage, when all embryonic structures are formed (Bentsink and Koornneef, 2002). The two fertilization products, embryo and endosperm, are surrounded by the seed coat, which ensures their protection throughout seed development and after the seeds being detached from the mother plant. The seed coat is originated from the ovule teguments, having only maternal origin (Figueiredo and Köhler, 2018). The growth period follows, in which the embryo fills up the seed sac, being the growth supported by the mother plant depositing nutrients in developing endosperm. In the end of embryo's growth, the cell division stops and the mature embryo contains the basic body plan of a future plant. The seed is so constituted by embryo, generally in dicots one layer of endosperm and the seed coat (Bentsink and Koornneef, 2002; Figueiredo and Köhler, 2018). Henceforth, the seed already containing a complete size, experiences maturation, a period when food reserve accumulation occurs and a tolerance to dormancy and desiccation is developed (Bentsink and Koornneef, 2002). The embryonic arrest and dormancy in seeds, during normal development, are finally reversed by the germination process, that occurs when favorable environmental conditions are supplied and the dry seed absorbs water (Bentsink and Koornneef, 2002; Figueiredo and Köhler, 2018).

This way seed metabolism is essential for physiological quality, germination and seedling establishment. Soon as imbibition occurs, there is a general reactivation of the metabolism to avoid oxidative stress, repair proteins, remobilize reserves, generate energy, start protein synthesis and produce biomass. All these mechanisms will influence the seed size, also susceptible to environmental variables and a crucial factor that determines crop yield (Domergue *et al.*, 2019; Lv *et al.*, 2019). It is also known that a high flux of N to juvenile flowers is essential for ensuring a high number of fruits with high number of seeds. Right after seed germination the seedling growth is also strongly influenced by N availability, being the N a key factor for seedling establishment with consequences in the cotyledons growth, storage reserve mobilization, chlorophyll levels and photosynthetic gene expression. (Guan *et al.*, 2015; Guan and Schjoerring, 2016; Fredes *et al.*, 2019). So, one way to reduce N fertilizer needs and increase productivity is to improve the N recycling and remobilization performances of plants and several studies have been devoted to the implications of these in grain yields and seed quality in the last decades (Bauer *et al*., 1997; Tabuchi *et al*., 2005; Thomsen *et al*., 2014; Moison *et al*., 2018).

The exact physiological functions of cytosolic GS isogenes in plant development, reproduction and seed productivity are still underexploited. Having the GS a critical role in primary N assimilation and amino acid metabolism, the loss of GS1 in several plant species often leads to the impairments of growth, development, and reproduction. Some

of the individual roles of GS1 isogenes of *A. thaliana* are acknowledged, but almost no data is available on their specific roles with respect to seedling development and seed yield (Lea and Miflin, 2011; Ji *et al.*, 2019).

During plant seed filling stage, the N remobilization occurs from vegetative tissues to seeds. So, leaf senescence and nutrient recycling provide some new perspectives for seed improvement, and GS plays an important role in these processes, being recent studies devoted to the implications of this enzyme in grain yields and seed quality (Lea and Miflin, 2011; Thomsen *et al.*, 2014; Guan *et al.*, 2015; Guan *et al.*, 2016; Konishi *et al.*, 2017; Konishi *et al.*, 2018; Moison *et al.*, 2018; Ji *et al.*, 2019). Senescence-induced degradation of leaf proteins in the reproductive growth stages, provides the main source for the N incorporated into seed storage proteins, and GS activity has been shown to correlate with this N remobilization efficiency (Guo *et al.*, 2004; Kichey *et al.*, 2005; Betti *et al.*, 2012; Guiboileau *et al.*, 2012; Guan *et al.*, 2015). It is also known that during seed reserve accumulation period, the glutamine levels in seeds are prominently reduced, signifying the incorporation of free amino acid into seed storage proteins. The metabolic changes occurring during this period in seeds are associated with gene expression changes (Fait *et al.*, 2006). Most of actual evidences for the role of cytosolic GS in nitrogen remobilization to grain filling has come from cereal studies of mutants deficient in cytosolic GS (Tabuchi *et al.*, 2005; Martin *et al.*, 2006). GS1 cereal mutants showed grain number and size of the plants directly affected by the determinant GS1 activity (Tabuchi *et al.*, 2005; Martin *et al.*, 2006; Funayama *et al.*, 2013), the *OsGS1;1* gene in rice revealed to be relevant in the grain filling (Funayama *et al.*, 2013). In *Arabidopsis thaliana* the *Gln1;2* gene plays an important role in N remobilization from vegetative parts during seed filling affecting seed production and seed yield (Guan *et al.*, 2015). Lately it was verified that *Gln1;1*, *Gln1;2*, and *Gln1;3* isogenes could offset each other in N remobilization for seed filling, being essential in this process. The studied triple mutant *gln1;1/gln1;2/gln1;3* ended up with a significantly lower seed yield (Moison *et al.*, 2018), and the *Gln1;4* isogene is described as for a senescence-specific GS1 (Lothier *et al*., 2011; Moison *et al*., 2018).

During seed germination, the storage protein turnover process generates large quantities of ammonium, subsequently reassimilated into glutamine to support growth and development of seedlings, and the GS role is certainly determinant in its assimilation and remobilization (Guo *et al.*, 2004; Betti *et al.*, 2012; Guan *et al.*, 2015; Wang *et al.*, 2015; Moison *et al.*, 2018; Ji *et al.*, 2019). In maize the *ZmGln1;3* and *ZmGln1;4* isogenes were already stated as essential for seed germination (Limami *et al.*, 2002; Orsel *et al.*, 2014) and in *A. thaliana* the *gln1;2* single mutants are impaired in seed germination and

affected in terms of N remobilization from cotyledons during germination (Guan *et al.*, 2016).

Nitrogen metabolism and GS, in particular, should also be very important during seed formation and development processes, although limited information is available. GS seed specificity occurred and so far, its role and importance weren't fairly explored, despite its pertinence in seed productivity (Bernard and Habash, 2009; Lea and Miflin, 2011; Dragićević *et al.*, 2014; Orsel *et al*., 2014; Domergue *et al*., 2019). On a sexual reproduction and seed development perspective the glutamine synthetase data is still scarce, not to say null, particularly for seed specific isogenes like *Gln1;5*. Besides the high specific expression at seed level, *AtGln1;5* appears to have also expression in flowers as a pollen specific GS (Schmid *et al.*, 2005; Soto *et al.*, 2010; Moison *et al.*, 2018). The *AtGln1;3* isogene, has also high expression in seeds and flowers. The role of these two genes in plant reproduction and seed formation is not yet explored and clarified (Lea and Miflin, 2011; Moison *et al.*, 2018). Therefore, GS research in physiology of seed development and the impact in seed performance are of outmost importance to significantly improve nitrogen use efficiency under the reduced fertilizer conditions that are needed for a sustainable agriculture and a healthy planet (Guo *et al.*, 2004; Fait *et al.*, 2006; Bernard and Habash, 2009; Betti *et al.*, 2012; Li *et al*., 2014; Guan *et al.*, 2015; Wang *et al.*, 2015; Ji *et al.*, 2019).

In 1991 Peterman and Goodman, while studying GS, thought that this gene family would represent a simple case in *Arabidopsis*, defining a minimum number of GS enzymes required by higher plants for nitrogen metabolism. Instead, they concluded that GS gene family revealed itself much more complex, being a great model system for differential and developmentally regulated gene expression study in higher plants (Peterman and Goodman, 1991). And so it is in fact, 28 years have gone by and the study of GS in *Arabidopsis thaliana,* particularly its seeds, is still an unknown path with much knowledge to be enlightened.

1.6. Objectives

The main purpose of this work is to study the role and importance of cytosolic glutamine synthetase isoenzymes in *Arabidopsis thaliana* seed development, and particularly of a seed specific isoenzyme, the GLN1;5. Presently, almost no data is available about *Gln1;5* gene, which expression is located in seeds with higher predicted expression. Therefore, one of the goals of this work is to understand and clarify the *AtGln1;5* gene role in seed development. The *AtGln1;3* role, the second GS1 isogene most expressed in seeds, will be also evaluated. These goals will be achieved through the characterization of mutants not expressing these GS1 isogenes and through expression studies approaches. To mislead the functional redundance possibility we will beget and study the *gln1;3*/*gln1;5* double mutant.

The results of this research will help to unravel and understand the cytosolic glutamine synthetase importance in *Arabidopsis thaliana* seed development and germination, contributing, perhaps, to future improvement of plant seed production.

2. Materials and methods

2.1. *In silico* analysis of the genes and mutant lines

The location of the T-DNA insertions for the mutant lines were verified and acquired from the SeqViewer tool, available in the TAIR website (www.arabidopsis.org).

The predicted expression patterns, qualitative assessment of the genes of interest and the possible interactions with other genes were verified through the eFP Browser, BAR ePlant platform (http://bar.utoronto.ca/eplant/, Waese *et al*., 2017).

The evolutionary relationships between the glutamine synthetase proteins were analyzed with MEGA X Software, (version 10.0.5, build 10180924, 1993-2019), (www.megasoftware.net, Kumar *et al.*, 2018). The evolutionary history of the generated tree was inferred by using amino acid change and the Maximum Likelihood method with JTT matrix-based. The reliabilities of each branch point were assessed by bootstrap analysis (10000 replicates).

The primers used in this work were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and PrimerIdent (http://primerident.up.pt/primerident_1.htm).

The plasmid transformations, restriction analysis and other related work were analyzed with SnapGene® software (version 5.0.6).

2.2. Plant material

In all experiments of this this work the ecotype Columbia (Columbia 0, [Col-0]) was used as a wild-type (Wt) *Arabidopsis thaliana* (L.). Wild-type, mutant lines *gln1;1* (SALK_000459), *gln1;3* (SALK_148604 and SALK_072283) and *gln1;5* (SALK_086579 and SALK_117504) seeds were obtained from the Nottingham Arabidopsis Stock Center (NASC), United Kingdom. The marker line plants for this work were produced as explained in section **2.6**.

The double mutant production was performed by cross fertilization. The closed flowers, on the development stage 12 as defined by Smyth *et al.* (1990), were emasculated 24 hours before the pollination with hypodermic needles (0.4 X 20 mm; B. Braun™) and protected with cellophane paper. The pollen used in the pollination was from mature dehiscent anthers. The cross breeding was performed with support of a Leica™ EZ4 Stereo Microscope and the crossings performed listed on **Table 1**. After fertilization the pistils were again protected with cellophane paper and the plants placed in the growth chamber.

Table 1 – Crossings performed with mutant lines.

gln1;5 (SALK_086579) ♀ X *gln1;3* (SALK_148604) ♂

2.3. Plant growth conditions

Seeds were sown on soil and remained in the dark at 4 °C for 48 hours to induce vernalization and then transferred to a growth chamber with long days photoperiod (16 hours of light and 8 hours of darkness), at a temperature of approximately 20 °C and 60% of relative humidity.

2.4. Selection of homozygous mutant plants

2.4.1. Genotyping

The genotypes of the mutant T-DNA insertion lines SALK_086579, SALK_117504, SALK_148604, SALK_072283, SALK_000459 were confirmed by polymerase chain reaction (PCR). The PCR was carried out with genomic DNA and using NZYTaq II 2x Green Master Mix (NZYTech™), a mix that contains all the reagents necessary for PCR, except primers and the DNA template. Two distinct reactions were performed for each SALK mutant line with the purpose to identify hetero- and homozygous mutants. Each reaction used a different combination of three primers: left primer (LP) and right primer (RP) are used to anneal to the forward and reverse strand of each gene, respectively, and the border primer (BP) anneals in the strand where is the T-DNA insertion border sequence present. The reaction with LP and RP combined (R reaction), amplified a section of the wild-type genomic sequence of the target gene, and the reactions with LP and BP or BP and RP (depending on the mutant line and in which strand the insertion is) (T reaction) amplified a fragment of the gene with the insertion. The T-DNA insertion in the genes on each mutant enlarges the size of the genomic sequence, then using LP and RP primers combined in the mutant does not amplify under the normal PCR extension conditions. The use of BP will promote a short size for the amplification fragment, this way the mutant plants were identified due to these fragments. LBb1.3 was the BP used on the SALK lines and the double mutant. The genotyping technique is shown in **Figure 4**. The primers used and the respective fragments size are in **Table 4**.

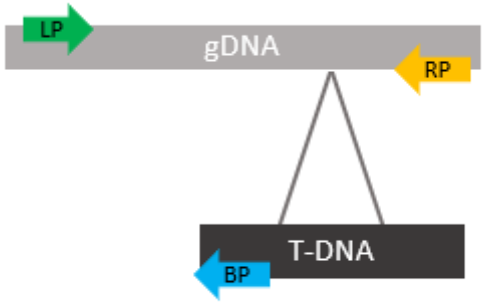


Figure 4 – Representation of the genotyping technique: The wild-type fragment is produced with an amplification using the left primer (LP) combined with the right primer (RP), producing a band when no T-DNA is present in the strand. However, when the T-DNA is present a mutant fragment will be produced when the border primer (BP) is combined with the left primer, as it is in the scheme.

2.4.2. Genomic DNA Extraction – CTAB method

Leaf tissues (100 mg) were homogenized and 300 µL of CTAB buffer [2% cetyl-trimethyl-ammonium bromide, 1% polyvinyl pyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA] were added. The plant tissues and buffer were thoroughly mixed by vortex and placed for 30 minutes at 65 °C, being frequently inverted. After the incubation period the homogenates were cooled to room temperature and 300 µL of chloroform/isoamyl alcohol [24:1] were added, and then completely vortexed. To promote separation of the phases the mixtures were centrifuged at 11400 g for 1 minute and the supernatant aqueous phases transferred to a new microtube. The DNA was then precipitated by adding 300 µL of isopropanol at 4 °C. To promote DNA precipitation the mixture was centrifuged at 11400 g for 5 minutes. The obtained pellet was washed with 70% ethanol and briefly centrifuged (30 seconds) at 11400 g. The gDNA was then diluted in 30 µL of ultra-pure H_2O (diH_2O) and stored at 4 °C.

2.4.3. PCR amplification

The PCR reactions were performed using NZYTaq II 2x Green Master Mix (NZYTech™) and the **Table 2** indicates the reagents and the concentrations used in the PCR amplification, a Bio-Rad™ T100™ Thermal Cycler was used and the PCR conditions for each *gln* mutant are on **Table 3**. The combination of primers used for the different amplifications, the specific oligonucleotides sequences and expected band sizes and reactions annealing temperatures are listed on **Table 4**.

Table 2 – Reagents and quantities used for PCR amplification.

Reagents	Quantity (Total of 20 µL)
Forward Primer (10 µM)	0.8 µL
Reverse Primer (10 µM)	0.8 µL
gDNA sample	1 µL
NZYTaq II 2x Green Master Mix	10 µL
diH_2O	7.4 µL

Table 3 – PCR conditions applied in each analysis with NZYTaq II 2x Green Master Mix (NZYTech™).

		PCR Programs		
Step		*Gln1;3*	*Gln1;1 & Gln1;5*	General
Initial denaturation		95 °C | 1 minute	95 °C | 1 minute	95 °C | 1 minute
35 Cycles	Denaturation	95 °C | 20 seconds	95 °C | 20 seconds	95 °C | 20 seconds
	Annealing	58 °C | 20 seconds	57 °C | 20 seconds	[55; 60] °C | 20 seconds
	Extension	72 °C | 60 seconds	72 °C | 45 seconds	72 °C | 1 Kb per minute
Final extension		72 °C | 5 minutes	72 °C | 5 minutes	72 °C | 5 minutes
Pause		4 °C | +∞	4 °C | +∞	4 °C | +∞

2.4.4. Gel electrophoresis

The amplified DNA fragments were separated in a 0.8% (w/v) agarose gel electrophoresis in 1X TAE buffer [0.04 M Tris-acetate, 0.001 M EDTA, pH= 7.6] and 0.03 µL/mL of GreenSafe Premium (NZYTech™) was added to the gel before polymerization. The molecular size marker used was the NZYDNA Ladder VIII (NZYTech™). The electrophoresis was performed in 0.25X TAE at 200 V with non-limiting amperage. The observations were performed and images acquired with a Bio-Rad™ ChemiDoc™ XRS (Bio-Rad Laboratories, Inc.) and analyzed with the Image Lab™ Software (version 6.0.1, build 34), standard edition (© 2017, Bio-Rad Laboratories, Inc.).

Table 4 – Complete list of primers used in this work (the plasmid adaptor sequences are shown underlined).

Name	Sequence (5' → 3')	Application	Annealing (°C)	Product size (≈ bp)
GLN1-1_FW_5UTR	CGTCACCCTCTTCCTCAATC	Genotyping *gln1;1* (SALK_000459)	60	800
AtGS1;1 (rev3)	GTAGCTGCGAAGGGTCAGT			
LBb1.3	ATTTTGCCGATTTCGGAAC			(LP + BP) 117
AtGS1;3(fwd2)	CGTCTTCTCTTTCCCTAAA	Genotyping *gln1;3* (SALK_072283)	57	1960
Gln1_3rev5	GTCTCAGCGATCATGGAGGT			
LBb1.3	ATTTTGCCGATTTCGGAAC			(BP + RP) 465
AtGS1;3(fwd2)	CGTCTTCTCTTTCCCTAAA	Genotyping *gln1;3* (SALK_148604)	57	1960
Gln1_3rev5	GTCTCAGCGATCATGGAGGT			
LBb1.3	ATTTTGCCGATTTCGGAAC			(LP + BP) 1830
Gln1_5fw	CATTACCAGGACCAGTAAGT	Genotyping *gln1;5* (SALK_086579)	58	661
AtGS1;5rev	CTTCACATTGGGATGATCG			
LBb1.3	ATTTTGCCGATTTCGGAAC			(LP + BP) 360
AtGS1-5_Fw3	GGCACAAGGCCGTAAAAATC	Genotyping *gln1;5* (SALK_117504)	55	357
AtGS1-5-Rev2	ACCAGGAAAGCCACCAAGA			
LBb1.3	ATTTTGCCGATTTCGGAAC			(LP + BP) 200
Atp_2049	CCACATGTTACGTCCTGTAGAAACCC	Genotyping *GUS*	60	620
Atp_0126	CACCACCTGCCAGTCAACAGACG			
Gln1;3ttB-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCT GTTAGTAGCCTGATGAAGTTATGACC	p*Gln1;3* amplification Gateway®	55	1211
Gln1;3ttB-Rv	GGGGACCACTTTGTACAAGAAAGCTGGGT CGGCGTCGGAGAGAAAATA			
Gln1;5ttB-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATCTCAGTGTTTAGTGATTGTTAGATG	p*Gln1;5* amplification Gateway®	55	634
Gln1;5ttB-Rv	GGGGACCACTTTGTACAAGAAAGCTGGGT TTTTGTTCCGAAAGAGAAAGAA			
AtGS1;1(fwd)	CATCCAAACCCTTGGTATT	RT-PCR *gln1;1* (SALK_000459)	60	310
AtGS1;1 (rev2)	GTAGCTGCGAAGGGTCAGT			
AtGS1;3(fwd2)	CGTCTTCTCTTTCCCTAAA	RT-PCR *gln1;3* (SALK_148604 & SALK_072283)	57	1097
Gln1_3rev5	GTCTCAGCGATCATGGAGGT			
Gln1_5fw	CATTACCAGGACCAGTAAGT	RT-PCR *gln1;5* (SALK_086579)	56	317
AtGS1;5rev	CTTCACATTGGGATGATCG			
AtGS1;1(fwd)	CATCCAAACCCTTGGTATT	RT-qPCR *Gln1;1*	-	170
AtGS1;1(rev3)	GTAGCTGCGAAGGGTCAGT			
AtGS1;2(fwd)	TAACCTTGACATCTCAGAC	RT-qPCR *Gln1;2*	-	133
AtGS1;2(rev2)	CTTTGGAAGTTTTGATGGATCG			
Gln1_3_fw3	CAAGAAAGCGATAGGGAAGC	RT-qPCR *Gln1;3*	-	252
Gln1_3rev5	GTCTCAGCGATCATGGAGGT			
AtGS1-5(FW3)	GGCACAAGGCCGTAAAAATC	RT-qPCR Gln1;5	-	137
AtGS1-5-Rev2	ACCAGGAAAGCCACCAAGA			
RT_147	CTGTTCACGGAACCCAATTC	RT-qPCR *RUB1*	-	133
RT_148	GGAAAAAGGTCTGACCGACA			
RT_861	CTCAGGTATTGCAGACCGTATGAG	RT-qPCR *ACT8*	-	185
RT_862	CTGGACCTGCTTCATCATACTCTG			
pDONR207-fw	GTTAACGCTAGCATGGATCTC	Sequencing	-	-
pDONR207-rv	CCAGAGCTGCAGCTGGATGG			

2.5. Gene expression analysis

2.5.1. RNA extraction

2.5.1.1. RNA extraction – Leaves and flowers

Mature, but not senescent, *A. thaliana* leaves and inflorescences from wild-type and mutant plants were collected and the RNA isolated using PureZOL™ RNA Isolation Reagent following the manual guidelines (Bio-Rad Laboratories, Inc.).

2.5.1.2. RNA extraction – Seeds

The RNA extraction from *A. thaliana* seeds was performed using a protocol established by Oñate-Sánchez and Vicente-Carbajosa (2008), with some minor modifications.

The seeds – collected at distinct stages of development (heart shape, early torpedo, green seeds) with hypodermic needles (0.4 X 20 mm; B. Braun™) with support of a Leica™ EZ4 Stereo Microscope or germinated (24 hours and 72 hours after germination) in petri dishes with a 5 mM nitrogen concentration medium (**2.7.2**) – were submersed in 550 µL of cool extraction buffer [0.4 M LiCl, 0.2 M Tris pH: 8, 25 mM EDTA, 1 % SDS] in 1.5 mL RNase free microtubes, grounded and 550 µL of chloroform was added. The samples were vortexed during 10 seconds, and then centrifuged for 3 minutes at 16100 g and 4 °C. The supernatants were transferred to new microtubes, 500 µL of water-saturated acidic phenol was added, and after vortexed, 200 µL of chloroform was also added and mixed. After a new centrifugation for 3 minutes at 16100 g and 4 °C. The supernatants were transferred to new microtubes and 1/3 volume of 8 M LiCl was added and mixed. The samples were precipitated at -20 °C for 1 hour and centrifuged for 30 minutes at 16100 g and 4 °C. The pellets obtained were dissolved in 470 µL of diH_2O, and 7 µL NaAc [3 M, pH= 5.2] and 250 µL 96 % ethanol were added and mixed. A centrifugation for 10 minutes at 16100 g and 4 °C was performed to precipitate the carbohydrates. The supernatants were transferred to new microtubes and 43 µL of NaAc [3 M, pH= 5.2] and 750 µL of 96% ethanol were added. After mix, the tubes were placed at -20 °C for, at least, 1 hour. After that a centrifugation was made for 20 minutes at 16100 g and 4 °C, the pellets washed with 700 µL of 70% ethanol and centrifuged for 3 minutes at the prior conditions. The air-dry RNA was resuspended in 22 µL of diH_2O.

2.5.1.3. RNA extraction – Pollen

Sixty mature *A. thaliana* flowers were placed in cool microtube with 550 µL of extraction buffer [0.4 M LiCl, 0.2 M Tris pH= 8, 25 mM EDTA, 1% SDS] and shaken for 30 minutes at 200 rpm. At the end all the flowers were removed from the microtubes. A brief 3 minutes centrifugation at 8000 g was performed in order to deposit the suspended pollen. The RNA pollen extraction was then performed as in **2.5.1.2**.

2.5.2. RNA quantification and integrity

All the RNA samples were quantified and the purity was verified using the µDrop™ Plate in a Multiskan™ GO MicroPlate spectrophotometer and the results analyzed with SkanIt™ Software (Thermo Scientific™). The RNA samples were run in a RNase free 1% (w/v) agarose gel in an electrophoresis unit washed with oxygen peroxide and diH_2O, with 0.25X TAE buffer, to verified the integrity. RNA samples were stored at -80 °C.

2.5.3. cDNA synthesis

The RNA samples were treated with dsDNase (Thermo Scientific™) according to the manufacturer's instructions in order to remove the genomic DNA contaminations. Reverse transcription was performed using Maxima™ H minus cDNA Synthesis Master Mix (Thermo Scientific™) and the procedure was according the manufacturer's instructions.

2.5.4. Semi-quantitative RT-PCR

For the semi-quantitative RT-PCR analysis, PCRs were performed using the primers shown in **Table 4**. The PCR conditions shown in **Table 5** and the NZYTaq II 2x Green Master Mix (NZYTech™) and other reagents as in **Table 2**.

Table 5 – PCR amplification conditions applied in RT-PCR.

RT-PCR Program

Step		Temperature	Time
Initial denaturation		95 °C	5 minutes
35 Cycles	Denaturation	95 °C	20 seconds
	Annealing	*	20 seconds
	Extension	72 °C	2 Kb per minute
Final extension		72 °C	5 minutes
Pause		4 °C	+∞

*Annealing temperatures according **Table 4**.

2.5.5. RT-qPCR

The cDNA used for RT-qPCR was amplified by PowerUp™ SYBR® Green Master Mix (Thermo Scientific™) in an CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using the program shown in **Table 6**. The specific primers used were previously tested by performing calibration curves for each pair, and listed in **Table 4**. *ACT8* (AT1G49240) and *RCE1* (AT4G36800) were defined as reference genes, with stable expression, and for each particular condition analyzed (in 10 µL reaction volume using 400 nM of each primer) 3 technical replicates were made. The dissociation curves were acquired after the amplification to verify its specificity. The data were analyzed with CFX Manager™ Software (version 3.1) (© 2012, Bio-Rad Laboratories, Inc.), also used for cycle threshold (C_t), primers efficiency calculation and to determine the significance of differences between samples. To normalize the gene expression data between the different biological samples the ratio between Ct value obtained for each primer pair and the housekeeping genes (*ACT8* and *RCE1*) was calculated. The ΔΔCq data was analyzed applying a scale relative to the average of expression of the target genes.

Table 6 – RT-qPCR program.

RT-qPCR Program

Step		Temperature	Time
Initial denaturation		95 °C	3 minutes
40 Cycles	Denaturation	95 °C	5 seconds
	Amplification	60 °C	3 seconds
Melt curve		[55; 95] °C	0.5 °C increments per second

2.6. *Gln1;3 and Gln1;5* marker lines production

2.6.1. Construct design

The constructs of the *GUS* reported gene fused to the *Gln1;3* and *Gln1;5* promoters (p*Gln1;3:GUS* and p*Gln1;5:GUS*) were created by using the Gateway® Gene Cloning system (Thermo Scientific™) (Hartley *et al*., 2000). Wild-type plants were transformed with constructs p*GLN1;3:GUS* and p*GLN1;5:GUS* by floral dip method (Clough and Bent, 1998), the seeds were harvested after the floral dip technique, sown and selected as in section **2.6.8**. Genotyping was made according section **2.6.9** and transgenic plants with the two different constructs were obtained.

The promoters of *Gln1;3* and *Gln1;5* genes were amplified from gDNA extracted of wild-type plants as described in **2.4.2**. The primers used were designed to amplify the -1213 to -2 region of the *Gln1;3* promoter and -634 to -1 region of the *Gln1;5* promoter. These

distances are relative to the initiation site (ATG nucleotide sequence). The amplification was realized using NZYTaq II 2x Green Master Mix (NZYTech™). The primers used for the amplification were: Gln1;3ttB-Fw and Gln1;3ttB-Rv for p*Gln1;3* amplification and Gln1;5ttB-Fw and Gln1;5ttB-Rv for the p*Gln1;5.* The primers were designed as in section **2.1** with the specific Gateway® adapting sequences (attB), shown underlined in the primers table (**Table 4**). The PCR parameters used for this amplification are described in **Table 7**.

Table 7 – PCR amplification parameters for promoter amplification with NZYTaq II 2x Green Master Mix (NZYTech™).

Promoter amplification PCR Program

Step		Temperature (°C)	Time
Initial denaturation		95	5 minutes
10 Cycles	Denaturation	95	30 seconds
	Annealing	55	45 seconds
	Extension	72	2 minutes
25 Cycles	Denaturation	95	30 seconds
	Annealing	68	45 seconds
	Extension	72	2 minutes
Final extension		72	5 minutes

The PCR products were separated in a 0.8 (w/v) agarose gel with 1x TAE buffer and the fragments of interest were excised and purified using NZYGelpure (NZYTech™) according the manufacturer's specifications. The bands excised were diluted in diH_2O and stored at -20 °C.

The BP reaction was performed with 5 µL of each attB-PCR product previously obtained, 1 µL of the entry vector pDONR™207 (Invitrogen™, 15 – 150 ng, **Figure S1**) and 2 µL of diH2O. Following 2 µL of Gateway® BP Clonase™ II Enzyme Mix (Thermo Scientific™), previously kept on ice for 2 minutes, were added and the mixture vortexed briefly and placed overnight at 25 °C for incubation. On the following day 1 µL of ice-cold proteinase K was added and the mix incubated for 10 minutes at 37 °C to inactivate BP Clonase™ enzyme.

The LR reactions were performed by adding 1 µL of the transformed plasmids, 1 µL of the destination vector pBGWFS7,0 (Gateway® , 15 – 150 ng, **Figure S2**) (Karimi *et al.*, 2002), 6 µL of diH_2O and 2 µL of vortexed Gateway® LR Clonase™ II Enzyme Mix (Thermo Scientific™), then mixed and incubated overnight at 25 °C. The next day, 1 µL of proteinase K was added into the mixture and incubation for 10 minutes at 37 °C was carry out to inactivate the enzyme.

2.6.2. Electrocompetent cells protocol – *E. coli* and *A. tumefaciens*

For the preparation of electrocompetent cells, colony of *E. coli* DH5α (or *A. tumefaciens* GV3101::pMP90 strain) was inoculated in 5mL of Luria-Bertani (LB) medium [1% (w/v) tryptone, 0.5% (m/v) yeast extract, 1% (m/v) NaCl, pH= 7.0] (for *A. tumefaciens* LB with gentamicin at 30 µg/mL and rifampicin at 50 µg/mL) and grown overnight at 37 °C with a 200 rpm agitation (at 28 °C with a 220 rpm agitation for *A. tumefaciens)*. Two milliliters (1 mL for *A. tumefaciens)* of culture were transferred to 200 mL of LB medium (for *A. tumefaciens* LB with gentamicin and rifampicin) and allowed to grow in the conditions previously reported, until the culture reach an optical density (OD) between 0.5 and 0.6. Then, the culture was placed in ice for 30 minutes. The *E. coli* culture was then divided in four portions of 50 mL (25 mL for *Agrobacterium*) and centrifuged for 20 minutes at 4000 g and 4 °C. After removed the supernatants each pellet was resuspended in 50 mL (25 mL) of cold diH_2O and centrifuged as previously reported. The supernatants were removed again and each pellet of bacteria resuspended on 25 mL (20 mL) of cold diH_2O. A last centrifugation took place at 4000 g and 4 °C and the pellets were dissolved in 2 mL (0.5 mL for *A. tumefaciens*) of 10% glycerol previously cooled in ice. Aliquots of 50 µL were made and stored at -80 °C.

2.6.3. Electrocompetent bacteria transformations

Aliquots of electrocompetent *E. coli* DH5α previously made or Agrobacterium tumefaciens (**2.6.2**) were used for each transformation using the pDONR™207 + *Gln1;3* promoter (**Figure S1A**) and pDONR™207 + *Gln1;5* promoter (**Figure S2A**). In each aliquot, after the bacteria thawed, was mixed 1.5 µL of either BP reaction or LR reaction (**2.6.1**), subsequently those bacteria were transferred to electroporation cuvette and placed in a Bio-Rad MicroPulser™ electroporator (Bio-Rad Laboratories, Inc.), where the adequate program (E.COLI [1.8 kV] or AGRO [2.2 kV] program, respectively) was applied. Immediately after the electroporation 300 µL of LB medium were added in the cuvettes, mixing gently with the pipette. The bacteria were then transferred to a microtube and kept for 1 hour at 37 °C (for *E. coli*) or at 28 °C for 4 hours (for *A. tumefaciens cells*), this without shaking, promoting transformed cells recovery.

After this period of rest 50 µL of each microtube solution were directly plated in Petri dishes with solid LB medium [with 1.6% (m/v) agar] and the specific antibiotics (**Table 8**). The remaining 300 µL were centrifuged for 2 minutes at 4000 g, 250 µL of the

supernatant discarded and in the remaining 50 µL the pellet resuspended by vortex and plated in the same terms.

E. coli cells were left growing overnight at 37 °C and *A. tumefaciens* cells grew at 28 °C for two days.

Table 8 – Antibiotics for each plasmid and cell type.

Cell	Plasmid	Antibiotic
Escherichia coli	pDONR™207	Gentamycin: 15 µg/mL
Escherichia coli	pBGWFS7,0	Spectinomycin: 50 µg/mL
Agrobacterium tumefaciens	pBGWFS7,0	Gentamycin: 30 µg/mL Rifampicin: 10 µg/mL Spectinomycin: 50 µg/mL

2.6.4. Liquid cell cultures

To proceed with plasmid isolation and get glycerol stocks of the cells successfully transformed, liquid cultures were made and grown. The colonies were taken from the Petri dishes with sterile toothpicks and placed in 15 mL tubes with 5 mL of LB medium inside, using the appropriated antibiotics in the correct concentration (**Table 8**). The *E. coli* were grown overnight at 37 °C, agitating with 200 rpm and *A. tumefaciens* GV3101::pMP90 for 48 hours at 28 °C with 220 rpm. Glycerol stocks were prepared with the cultures of interest by adding glycerol to the liquid cultures, stocks of 20% glycerol were made and preserved at -80 °C.

2.6.5. Minipreps of plasmid DNA

For the plasmid DNA extraction 2 mL of bacterial culture were centrifuged at 12000 g and the supernatant discarded. The plasmid DNA was isolated using the NZYMiniprep kit (NZYTech™) according to the manufacturer's manual.

2.6.6. Restriction analysis

The constructs were verified by digestion with restriction enzymes using SnapGene® (v.5.0.6) to select the adequate enzymes. The pDONR™207 constructs were verified using the EcoRV enzyme and the pBGWFS7,0 using the SmaI. For the digestion 5 µL of plasmid, 2 µL of BSA, 2 µL of adequate buffer, 1 µL of enzyme and 10 µL of diH_2O were mixed and incubated overnight at 37 °C (for EcoRV) and 25 °C (for SmaI).

2.6.7. Sequencing

Sequencing was performed by STAB VIDA. The received data by the enterprise was aligned and analyzed using the Clustal Omega online platform (https://www.ebi.ac.uk/Tools/msa/clustalo/). The primers used were pDONR207-fw and pDONR207-rv (**Table 4**).

2.6.8. *A. thaliana* plants transformation by floral dip technique

Arabidopsis plants (wild-type ecotype Col-0) with 1 month and a half old were transformed by the floral dip technique following the established protocol by Clough and Bent (1998). Isolated transformed colonies *of A. bacterium* GV3101::pMP90, of each transformation, pBGWFS7,0 + *Gln;3* promoter (**FigureS1B**) and pBGWFS7,0 + *Gln1;5* promoter (**Figure S2B**), were picked and inoculated in 5 mL of LB medium supplemented with 10 µg/mL rifampicin, 30 µg/mL gentamycin and 50 µg/mL spectinomycin. The inoculums were incubated at 28 °C for 2 days with a 220-rpm shaking. The cultures were then centrifuged at 5500 g, room temperature, for 20 minutes and the pellets resuspended in 250 mL solution with 5% (m/v) sucrose and 0.05% (v/v) Silwett-77 (a detergent to reduce water surface tension, increasing the contact surface of the solution with the plant). The buds of the plants were then dipped in this solution for 2 minutes, twice, and with 1 minute of pause between each dipping. The plants were covered with plastic bags during 12 hours and placed in the growth chamber.

The *Arabidopsis* seeds were collected when the plants were completely dry and kept in obscurity at 4 °C for 48 hours for tissue stratification, then imbibed in water for 48 hours for vernalization. The seeds were finally sown on soil and left to grow, later, the seedlings were selected with a glufosinate ammonium herbicide, Basta® (10 mg/L). The treatment was applied once every two days in a whole of 3 times. The resistant plantlets, those which maintained a vivid green color, were transplanted into individual pots.

2.6.9. Transgenic plants genotyping

The genotyping of the plants selected by Basta® treatment was performed using the Atp_2049 (LP) and Atp_0126 (RP) primers (**Table 4**), specific for *GUS* detection. The presence of an approximately 620 bp band indicated the presence of the gene in the transformed plants. More on genotyping in section **2.4**.

2.6.10. GUS activity - Histochemical detection

Fresh plant material was collected to 0.2 µL microtubes with 90% acetone (fixative) and incubated overnight at -20 °C. The samples were then washed 2 times with sodium phosphate (NaPi) buffer [0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4] for 5 to 10 minutes. The next steps of this method were always performed protecting the samples from the light. The buffer was then replaced by a X-Gluc (5-bromo-4-chloro-3-indolylglucuronide) solution [50% phosphate/NaPi buffer, 1% Triton X-100, 0.8% potassium ferrocyanide ($K_4[Fe(CN)_6]\cdot 3H_2O$), 0.8% potassium ferricyanide ($K_3[Fe(CN)_6]$), 46.4% diH_2O, 100% (w/v) X-Gluc] and left overnight in the incubator at 37 °C. The X-Gluc solution was removed and the samples were washed with 90% ethanol and then with 70% ethanol for 10 minutes each. Finally, the ethanol was discarded and the biologic material submersed in chloral hydrate and left at 4 °C overnight.

The results were observed using a Zeiss™ Axiolmager AZ microscope with differential interference contrast (DIC) optics and photographed with a Zeiss™ Axiocam MRc3 camera through the Zen Imaging acquisition software (SP1, Zen 2011).

2.7. Phenotype characterization assays

2.7.1. Pollen Essays

2.7.1.1. Aniline blue staining

Mutant and wild-type pistils from stage 12 flowers (Smyth *et al.,* 1990) were emasculated and 24 hours later were hand pollinated, as described in section **2.2**, with mutant and wild-type pollen (**Table 9**). After 8 hours the pistils were collected and fixed in an absolute ethanol and acetic acid solution [3:1] and left overnight at 4 °C. The biologic samples were washed 3 times with water for 5 minutes each time and then it was applied for clearing a solution of NaOH [1 M] overnight. Next 3 washes were performed with water for 30 minutes to 1 hour each, and finally the samples were submersed in a 0.1% (w/v)

aniline blue solution at 4 °C. The observations were made under ultraviolet light using an upright Eclipse Ti-S (Nikon™) microscope and the images captured with a ProgRes® MF (Jenoptik™) camera and the NIS-Elements Basic Research Software (Nikon™).

2.7.1.2. Alexander staining

Pollen from mutant and wild-type plants was collected in non-dehiscent anthers, just before anthesis, from flowers with an estimated development stage 15 as defined by Smyth *et al.* (1990). The anthers were removed from the flowers using forceps, with the help of a Leica™ EZ4 Stereo Microscope and placed in a some drops of Alexander stain solution [10 mL 95% ethanol, 5 mL of 1% malaquite green in ethanol 95%, 5 mL 1% acid fuchsin, 0.5 mL 1% orange G, 2 mL glacial acetic acid, 25 mL glycerol, 5 g phenol, up to 100 mL H_2O], gently shaken to release the pollen grains into the solution. The counting of the pollen was performed using a Zeiss™ Axiolmager AZ microscope with DIC optics and photographed with a Zeiss™ Axiocam MRc3 camera through the Zen Imaging acquisition software (SP1, 2011 ZEN).

2.7.1.3. Pollen tubes culture

The pollen of wild-type and mutant plants was collected from flowers in stage 15 of development (Smyth *et al.*, 1990), and per replicate (3 technical replicates of the same pollen type) 40 fresh opened flowers were placed in a 1.5 mL microtube and then gently vortexed with 1mL of pollen growth medium [5vmM KCl, 5 mM CaCl2, 1 mM MgSO4, 0.01% boric acid, 10% sucrose, pH= 7.5] for 5 minutes. The flowers were removed from the tube with the help of forceps. The microtubes were then centrifuged at 3000 g for 3 minutes and the supernatant discarded. The pelted pollen of the same type was grouped (a total of 120 flowers – 3 microtubes) and suspended in 1.25 mL of fresh growth medium. The pollen was incubated at 22 °C in the dark for 2, 4 and 6 hours. Pollen tube growth was assessed by the number of germinated pollen grains at the established times and the counting results (100 pollen grains per technical replica) were observed using a Zeiss™ Axiolmager AZ microscope and photographed with a Zeiss™ Axiocam MRc3 camera through the Zen Imaging acquisition software (SP1, 2011 ZEN).

2.7.2. Germination, growth and fresh weight assays

For the germination and growth assays the seeds were germinated at 3 different nitrogen concentrations: 0 mM, 5 mM and 20 mM. The Hoagland medium used in the assays had some adaptations in order to be possible to control specifically the nitrogen

concentrations. Each assay was performed with a total of 18 sterile petri plates (12.5cm X 12.5 cm), 3 replicates for each N concentration, with 50 mL of growth medium [1 mL of 1 M phosphate buffer pH= 7, 0.1 mL of 2 M $CaCl_2$, 0.1 mL of 0.5 M $MgSO_4$, 0.2 mL of 50 mM Fe/EDTA, 0.4 mL Hoagland micronutrients, 0.3 mL of 0.35 mM K_2SO_4 (for 0 mM nitrogen), 0.375 mL of 1 M KNO_3 (for 5 mM nitrogen), 1.5 mL of 1 M KNO_3 (for 20 mM nitrogen), up to 200 mL H_2O, pH 5.8, 1.6% agar]. Each plate had a total of 20 seeds that were previously vernalized for 48 hours at 4 °C in obscurity and disinfected with a bleach solution [0.25 mL 10% SDS, 1 mL bleach, up to 10 mL of diH_2O] for 15 minutes. Seeds were washed with diH_2O and transferred to the plates with sterile toothpicks, and the plates sealed with micropore tape. The plates went finally to a growth chamber under long days photoperiod (16 hours of light per day) at 20 °C and 60% of relative humidity. The measures of the radicles and seedlings were made in the 5th, 10th and 15th days under a Leica™ EZ4 Stereo Microscope. The seeds were considered germinated when the emergence of the radicle from the seed was visible.

After finished the growth assays the plants were weighted, at the 15th day, to determine their fresh weight. The fresh weight assay had three technical replicates, each one with ten plantlets.

The germination assay for the double mutant seed viability scrutiny was performed only at a nitrogen concentration of 5 mM. Five technical replicates were used with 40 seeds per replicate. The germinated seeds were counted 5 days after sown.

2.7.3. Visual characterization assay

The phenotype characterization of the mutant plants was also performed by their visual analysis. The number of leaves were counted, when the floral stems appeared, in which number and their length. The number of flowers in plants with one month old and the number of siliquae at one month and a half after sowing were also counted. These assays were always made comparing the mutant plants with wild-type. The plants were grown in a growth chamber as defined in section **2.3**.

2.7.4. Seed set analysis

The mutant seeds were compared to the wild-type to observe any phenotypical differences. The assays took at least 20 siliques, the last 5 siliquae of each stem of different replicates, for the seed set analysis. All the siliques were measured with a millimetric scale and then dissected with hypodermic needles (0.4 X 20 mm; B. Braun™) to analyze their seeds, discriminated in green seeds, white seeds, aborted seeds and

aborted ovules. The observations were performed through a Leica™ EZ4 Stereo Microscope.

2.7.5. Seed morphology assay

For all the homozygous mutants studied in this work comparative observations of the mutant seeds with the wild-type ones were performed. The observations were made under a Leica™ EZ4 Stereo Microscope comparing the morphology of the seeds, existing 3 major phenotypes: round seeds (regular, without defects), flat seeds (collapsed ones) and shriveled seeds (problems separating the endosperm and embryo), (Yang *et al*., 2007). For each line 20 seeds were counted and this assay was performed 3 times.

2.8. Image processing

The images of this work were processed using the software Image J (Schneider *et al.*, 2012).

2.9. Statistical Treatment

All the assays of this work that produced data subjected to statistical treatment recurred to GraphPad Prism® (version 6.01, 1992 - 2012). The studies were made through one-way ANOVA tests or t-student tests with Welch's correction, when needed. All the assays had at least 3 replicates for each sample and the tissues (when necessary) for each assay pooled from plants growing in the same conditions.

3. Results and discussion

3.1. *In silico* analysis of the *A. thaliana* GS genes

3.1.1. GS genes expression patterns in *A. thaliana*

In order to analyze which of the glutamine synthetase genes have pertinent expression at seed level, and proceed to their future study, an *in silico* analysis was performed. The predicted expression patterns of the glutamine synthetase genes in *A. thaliana* are available in the eFP Browser platform (http://bar.utoronto.ca/eplant/). The results derived from microarray data compilation give the expression pattern of the different genes. According to the platform, in *A. thaliana*, all the glutamine synthetase genes have distinct expression patterns, suggesting specific roles for each protein.

The *Gln1;1* expresses mainly on cauline and senescent leaves (also in their petioles and veins) and in flower protection organs, like petals and sepals, with higher values in stage 15 of flower development. It has also expression in the roots (**Figure 5A**) corroborating several reported works (Ishiyama *et al*., 2004; Dragićević *et al*., 2014; Guan *et al*., 2015; Guan and Schjoerring, 2016; Moison *et al*., 2018). The female flower structures and mature pollen do not to appear to show *Gln1;1* expression (**Figure 5B**). During seed formation the expression of *Gln1;1* has medium values and increases in the last maturation stages (**Figure 5A**), expressing mainly on the seed coat and the peripheral endosperm (**Figure S1A**). The torpedo phase seems to have the lower values of expression. During seed germination the expression of the *Gln1;1* increases in the second soaking day, having a slight expression decrease in the third day, denoting some relevant role on the early phases of germination (**Figure 5A**).

The *Gln1;2* is the GS1 gene more widely expressed in *A. thaliana* and with higher expression in vegetative plant tissues, leaves and roots. In the male structures the *Gln1;2* expression is higher in young and mature flowers, having the mature pollen low expression values. It has expression in the petals and sepals and low expression values in the female gametophyte (**Figure 5C**). During seed formation (**Figure 5C**) the *Gln1;2* expression decreases and it is expressed in the seed coat, mainly in chalazal seed coat (**Figure S1B**). During germination (**Figure 5D**) the expression slightly increases. In the seedlings *Gln1;2* expression is higher in the root (**Figure 5D**). The wide expression and lower values in the mature pollen and seeds corroborate the work of Schmid *et al.* (2005) and Lothier *et al.* (2011).

The *Gln1;3* gene main expression is related to flowers, buds and seeds (**Figure 5E**). The expression in the male structures of the flower is higher at stage 12 of flower development and decreases until its maturation (**Figure 5F**). The analysis predicts *Gln1;3* expression in the roots as already described in Konishi *et al.* (2017) and Moison *et al.* (2018) and in senescent leaves (**Figure 5E**).

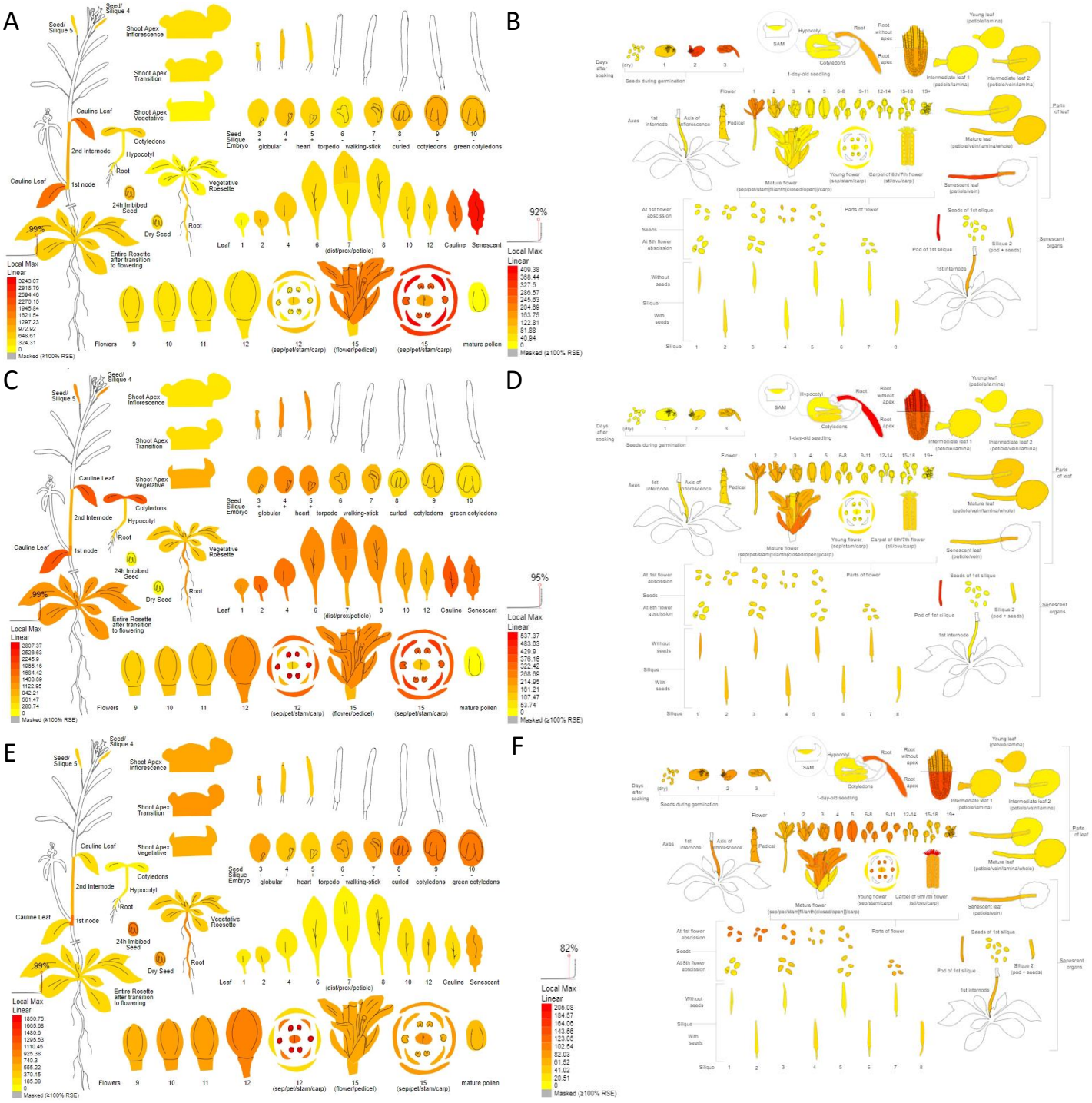


Figure 5 – Expression pattern of cytosolic glutamine synthetase (GS1) isogenes in *Arabidopsis thaliana*, according the platform ePlant (http://bar.utoronto.ca/eplant/, Waese *et al.*, 2017). **A, B –** *Gln1;1* (At5g37600); **C, D –** *Gln1;2* (At1g66200); **E, F –** *Gln1;3* (At3g17820).

This isogene is highly expressed in floral parts in all stages of development, in structures like pedicel, petals and sepals, and also in the reproductive organs. It is expressed in the carpel and ovules, being the stigma the structure with higher values in the feminine flower organs (**Figure 5F**). The siliqua development has a decreasing expression of the *Gln1;3* (**Figure 5E**) and during the seed formation the expression is high with increasing values during the maturation process (**Figure 5E**). The expression occurs in the seed coat and in the mature green seeds' embryo (**Figure S3C**), while during germination the expression values just increase slightly. In seedlings the expression is higher at a root level (**Figure 5F**).

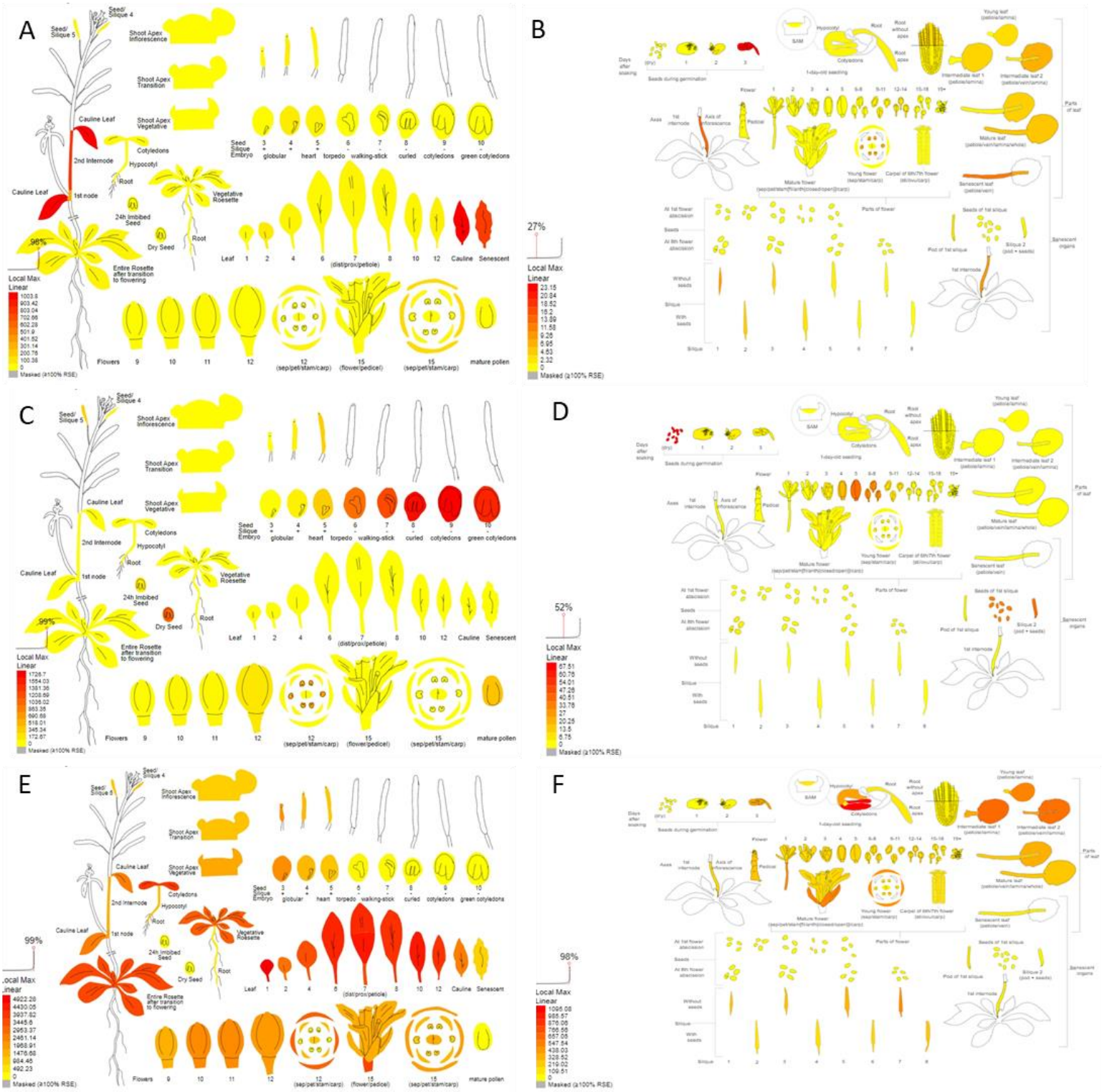
The *Gln1;4* expression seems to be restricted to senescent leaves (the senescent petioles and veins also) and this particular pattern of expression has already been reported (Bernhard and Matile, 1994; Guo *et al.*, 2004; Ishiyama *et al.*, 2004; Diaz *et al.*, 2008; Lothier *et al.*, 2011; Avilla-Ospina *et al.*, 2015) (**Figure 6A**). It is predicted to be expressed in cauline leaves, at the first and second internode (**Figure 6A**) and in seeds at the third soaking day (with high expression values) (**Figure 6B**). No expression is predicted during seed formation (**Figure 6A, Figure S3D**) or during germination. At flower level some expressions occur in the pollen of young flowers (**Figure 6B**).

The expression of *Gln1;5* gene seems to be absent from roots and rosettes, confirming the studies by Ishiyama *et al.* (2004) and Lothier *et al.* (2011), being the predicted expression pattern seed and pollen specific (**Figure 6C**) and without expression in the female gametophyte (**Figure 6C, 6D**). The expression in the pollen is low, having higher values in the stage 12 of the flower. In the seeds the expression has the highest levels, increasing during the seed maturation process (**Figure 6C**). Of all GS genes, *Gln1;5* is the one with the highest expression in mature seeds. *Gln1;5* is expected to express in the suspensor of the embryo at the seed's globular phase and in the embryo tissues from torpedo phase on (**Figure S3E**). During seed gemination the *Gln1;5* expression diminishes in the seedlings constituting tissues (**Figure 6D**).

The *Gln2* gene, the plastidic glutamine synthetase, has the major expression in green tissues, being the most expressed GS form in the green leaves (**Figure 6E**). In flowers the *Gln2* gene is expressed in all structures, particularly in the sepals of young flowers and has lower expression values in the male and female structures (**Figure 6E, 6F**). During embryogenesis the expression is moderated at globular embryo stage, but by the torpedo phase the expression has completely ceased (**Figure 6E**). It has high expression in the micropylar endosperm at the beginning of the seed formation (**Figure S3F**). During germination the expression appears at the third soaking day, and at day one seedlings its expression is higher in the cotyledons and moderated at hypocotyl level (**Figure 6F**).

This *in silico* study confirms the expression of the plastidic glutamine synthetase also in non-photosynthetic tissues, like already described in Woodall and Forde (1996), Orea *et al.* (2002) and Melo *et al.* (2003).

Figure 6 – Expression pattern of glutamine synthetase (GS) genes in *Arabidopsis thaliana,* according the platform ePlant



(http://bar.utoronto.ca/eplant/, Waese *et al.*, 2017). **A, B –** *Gln1;4* (At5g16570); **C, D –** *Gln1;5* (At1g48470); **E, F –** *Gln2* (At5g35630).

The main goal of the present work was to study the importance of GS during seed development and germination. From the exposed data the *Gln1;2 and Gln2* are not interesting genes to study as they present low and decreasing expression during the seed formation and only moderated increased expression during germination. The *Gln1;4* gene seems to have a role in seed formation, being the less interesting target gene. The *Gln1;3* and *Gln1;5* genes revealed to be the most appealing targets to start a GS study on *A. thaliana* seeds. Both genes have high expression values during the seed formation, and their expression increases during seed maturation, suggesting a possible relevant role in embryo development and seed maturation process. As previously mentioned, *Gln1;3* and *Gln1;5* also have expression in the reproductive floral parts (**Figure 5E**, **5F**, **6C**), making these genes also interesting on a plant sexual reproduction perspective. A third GS gene, also defined to a possible seed development study, is the *Gln1;1* which expression in seeds is balanced and moderated through all the seed formation process, but having a huge increase during the initial stages of germination.

3.1.2. Possible protein interactions

In order to understand the interactions of the selected GS genes (*Gln1;3* and *Gln1;5*) with other proteins, the ePlant platform was used to select the interaction viewer. The *Arabidopsis* interaction viewer uses a database of 79,793 predicted and 62,529 (to date) experimentally determined interacting proteins (http://bar.utoronto.ca/eplant/). The analysis of all predicted interactions for both these proteins (**Figure 7**) allowed to elaborate the **Table 9** in which the putative genes of interest having activity in the seed formation and/or germination processes and interacting with *Gln1;3* and *Gln1;5* are displayed.

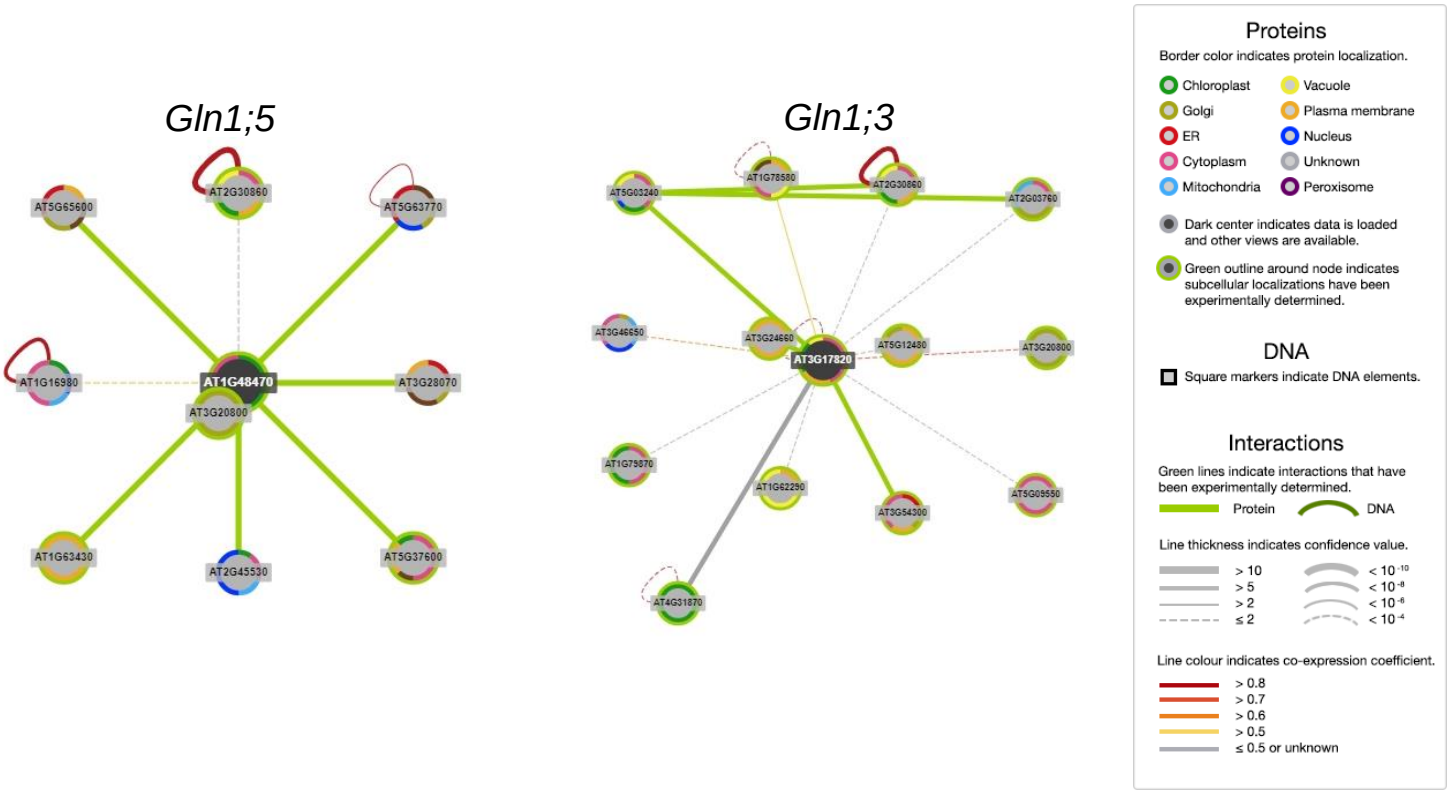


Figure 7 – *In silico* predicted interactions of *Gln1;3* (At3g17820) and *Gln1;5* (At1g48470), isogenes proteins with other *Arabidopsis thaliana* proteins. Data collected from the platform ePlant (http://bar.utoronto.ca/eplant/, Waese *et al.*, 2017).

Table 9 – Protein interactions with *Gln1;3* and *Gln1;5* in *Arabidopsis thaliana* and expressed in seeds.

Gene	Description	Biological Process	Expression Local	Interacts with
At1g62290	Saposin-like aspartyl protease family protein	Lipid metabolic process, protein catabolic process, proteolysis	Seedling; Seeds	*Gln1;3*
At1g63430	Leucine-rich repeat protein kinase family protein.	Protein phosphorylation	Pollen; Seedling; Seeds	*Gln1;5*
At1g79870	D-isomer specific 2-hydroxyacid dehydrogenase family protein	Oxidation-reduction process, oxidative photosynthetic carbon pathway	Pollen; Seedling; Seeds	*Gln1;3*
At2g03760	Sulphotransferase 12	Brassinosteroid metabolic process, defense response, response to salicylic acid, response to salt stress	Seedling; Seeds	*Gln1;3*
At2g30860	Glutathione S-transferase PHI 9	Defense response, glutathione metabolic process, oxidation-reduction process, response to cadmium ion, response to zinc ion, toxin catabolic process	Seedling	*Gln1;3 & Gln1;5*
At2g45530	RING/U-box superfamily protein	Zinc ion binding	Pollen; Seedling; Seeds	*Gln1;5*
At3g20800	Cell differentiation, Rcd1-like protein	Negative regulation of translation	Pollen; Seedling; Seeds	*Gln1;3 & Gln1;5*
At3g24660	Transmembrane kinase-like 1	Protein phosphorylation	Pollen; Seedling; Seeds	*Gln1;3*
At3g54300	Vesicle-associated membrane protein 727	Protein targeting to vacuole, vacuole organization, vesicle-mediated transport	Pollen; Seedling; Seeds	*Gln1;3*
At4g31870	Glutathione peroxidase 7	Oxidation-reduction process, positive regulation of defense response to insect, response to karrikin, response to oxidative stress	Dry seeds	*Gln1;3*
At5g03240	Polyubiquitin 3	Modification-dependent protein catabolic process, protein ubiquitination, response to UV-B, ubiquitin-dependent protein catabolic process	Pollen; Seedling; Seeds	*Gln1;3*
At5g12480	Calmodulin-domain protein kinase 7	Intracellular signal transduction, peptidyl-serine phosphorylation, protein autophosphorylation	Pollen; Seedling; Seeds	*Gln1;3*
At5g37600 (*Gln1;1*)	Glutamine synthetase clone R1	Glutamine biosynthetic process, leaf senescence, nitrate assimilation	Seeds	*Gln1;5*

The results in **Table 9** show 13 different interesting genes, 10 of which interact with *Gln1;3* and 5 with *Gln1;5, and* 2 of these candidates interact with both genes. The *Gln1;1* is one of the identified genes, the correspondent protein interacts with the *Gln1;5* gene.

This interaction reinforces the importance of *Gln1;1* as an appealing target to be studied at seed level.

The interactions observed involve proteins with biological roles in brassinosteroid metabolism, defense responses, lipid metabolism, negative regulation of translation, protein catabolism, protein phosphorylation, stress responses, among others. This variety of interactions stresses the idea of the multiple processes in which the GS genes can be involved and open new avenues to future studies.

3.1.3. Glutamine synthetase proteins: evolutionary relationship

For a better framing of the different GS proteins of *A. thaliana* in an evolutionary perspective a phylogenetic approach was performed recurring to MEGA X software, generating the results in **Figure 8**.

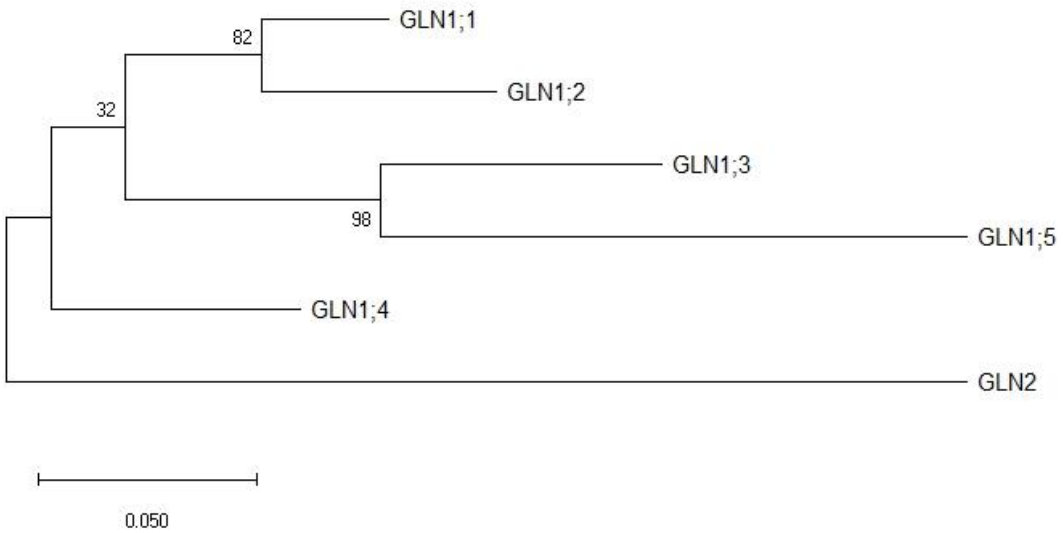


Figure 8 – Unrooted phylogenetic tree of GS protein sequences from A. thaliana. Analysis was carried out using MEGA X: molecular evolutionary genetics analysis across computing platforms software. The reliabilities of each branch point were assessed by bootstrap analysis (10000 replicates). Bootstrap values are displayed on the tree (Kumar *et al*., 2018).

The GS genes more highly expressed in seeds, *Gln1;3* (**Figure 5E**) and *Gln1;5* (**Figure 6C**), and the main targets of this study, have the highest sequence similarity, being grouped in the phylogenetic analysis (**Figure 8**). The sequence encoding chloroplastic GS formed an isolated clade from the GS1 sequences and the results of this phylogenetic tree corroborate the data from Bernard *et al.* (2008), for the *Arabidopsis* GS proteins.

3.2. Mutant lines genotyping

From the data analysis in the previous chapters two GS1 genes (*Gln1;3* and *Gln1;5*) were selected to further study in order to decipher the role of GS in seed development and germination. To examine the effect of a loss of function of the selected genes, homozygous T-DNA insertion mutants were isolated and analyzed.

In order to identify the homozygous mutant plants a two genomic PCR reactions approach was performed. One reaction was performed using the border primer (BP), that binds to the border of the T-DNA insertion, and the left primer (LP), which binds to the reverse strand of the target gene. The amplification with BP and LP (T reaction) detects the presence of the insertion. The other reaction used two gene-specific primers that encompass the T-DNA insertion site, the LP, and the right primer (RP), that binds to the forward strand of the target gene, and only amplifies the wild-type DNA. The homozygous plants were selected only when the amplicon from the first reaction was detected. The genes scheme and the positions of the T-DNA inserts of each mutant line used are represented in **Figure 9**. Two insertion lines were analyzed for *Gln1;3* (SALK_072283 and SALK_148604) and *Gln1;5* (SALK_086579 and SALK_117504), one for *Gln1;1* (SALK_000459).

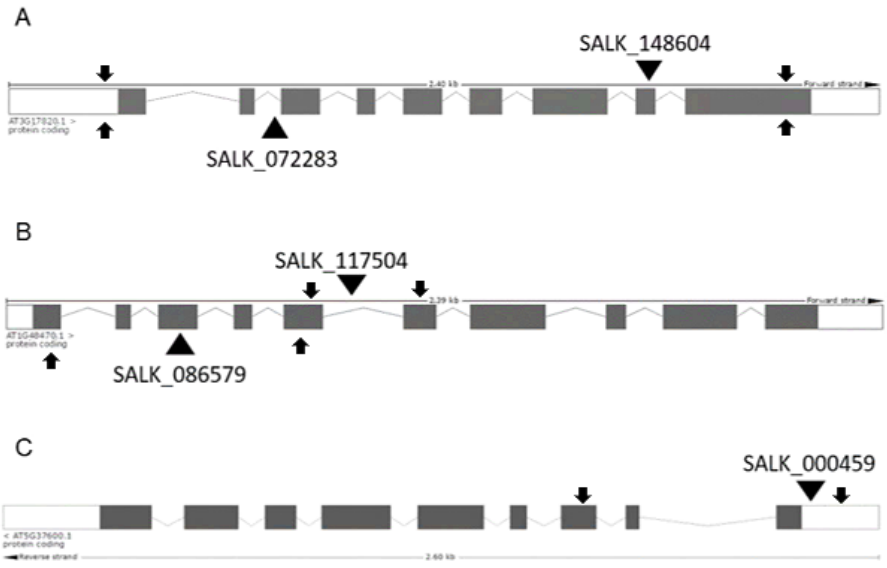


Figure 9 – Representative scheme of the target genes studied in this work. The exons are represented by the grey boxes and the introns by the lines. The position of the T-DNA insertion of the mutant SALK lines are identified in the scheme and the approximated position of the primers used in genotyping is indicated by the arrows. **A –** *Gln1;3* gene (At3g17820); **B –** *Gln1;5* gene (At1g48470); **C –** *Gln1;1* gene (At5g37600). (The graphic images were adapted from http://plants.ensembl.org/.)

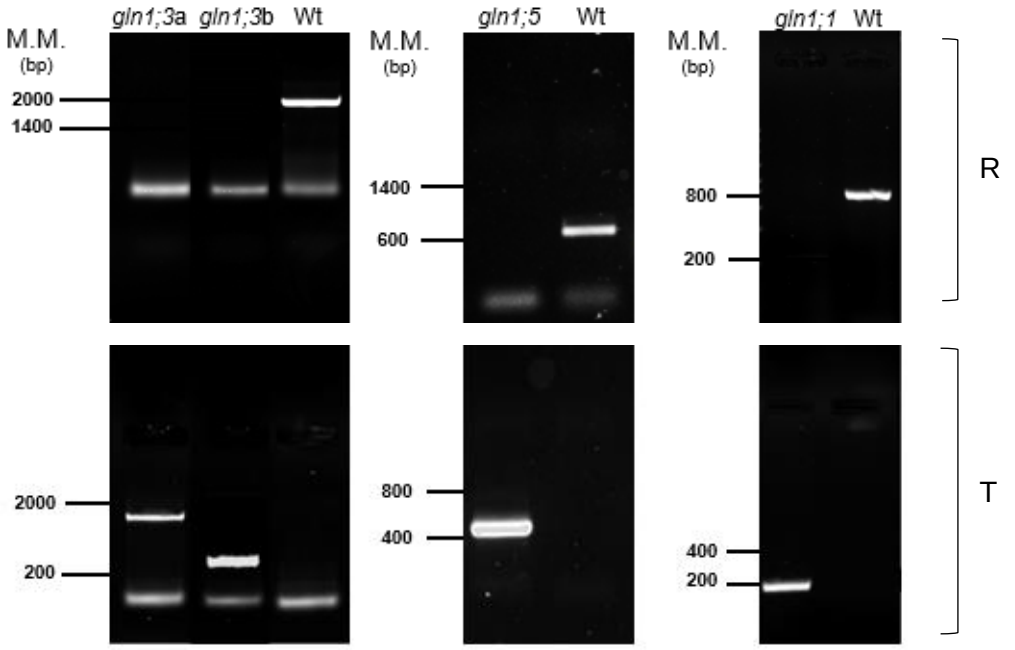


Figure 10 – Electrophoresis corresponding to the mutant lines genotyping results. Wt – Wild-type plant; ***gln1;3*a –** SALK_148604 mutant line; ***gln1;3*b –** SALK_072283 mutant line; ***gln1;5* –** SALK_086579 mutant line ; ***gln1;1* –** SALK_000459 mutant line; **R –** R reaction (wild-type fragment); **T –** T reaction (insert fragment); **M.M. –** Molecular size marker.

Four mutant lines, in a total of 5 in this work, were successfully genotyped (**Figure 10**). In SALK_148604 (**Figure 10**: *gln1;3*a) and SALK_072283 (**Figure 10**:*gln1;3*b) homozygous mutants, the wild-type fragment with the expected size of approximately 1960 bp was absent and the T-DNA fragments, with the expected size of 465 bp and 1830 bp, respectively, were detected. In *SALK_086579* homozygous mutant (**Figure 10**: *gln1;5*) the wild-type fragment with an expected size of 661 bp was absent, and the insert fragment with a predicted size of 360 bp was detected. Finally, for the *SALK_000459* homozygous mutants (**Figure 10**: *gln1;1*) the wild-type fragment with the predicted size of 800 bp was not generated, and the insert fragment with the expected size of approximately 117 bp was amplified.

The knockout expression of the *gln1;3*, *gln1;5* and *gln1;1* mutants was confirmed using a semiquantitative RT-PCR approach. The cDNA from the flowers of homozygous mutant plants SALK_148604 (**Figure 10**: *gln1;3*a), SALK_072283 (**Figure 10**: *gln1;3*b), and SALK_086579 (**Figure 10**: *gln1;5*), and leaves of SALK_000459 (**Figure 10**: *gln1;1*) T-DNA insertion homozygous mutant plants were used in the analysis (**Figure 11**). The primers used are indicated in **Table 4,** amplifying a 1097 bp, 317 bp and 310 bp fragments, respectively for *Gln1;3*, *Gln1;5* and *Gln1;1* when the genes are expressed.

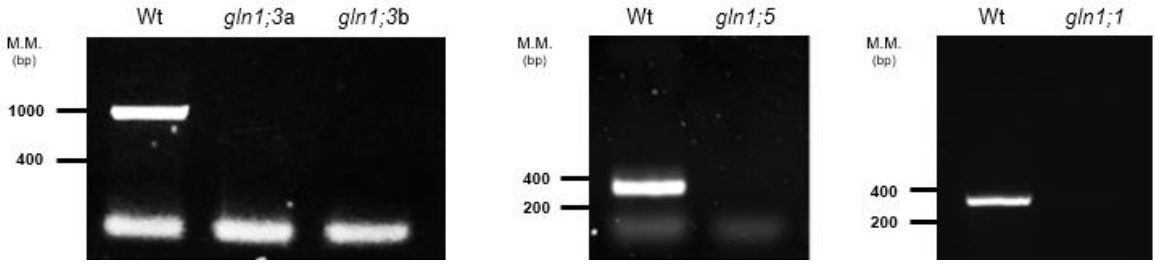


Figure 11 – Electrophoresis corresponding to the results of RT-PCR analysis of the mutant plants. **Wt –** Wild-type plant; ***gln1;3*a –** SALK_148604 mutant line; ***gln1;3*b –** SALK_072283 mutant line; ***gln1;5* –** SALK_086579 mutant line; ***gln1;1* –** SALK_000459 mutant line; **M.M. –** Molecular size marker.

The results (**Figure 11**) show that the GS genes are expressed only in the wild-type plants and the four mutant lines are knockout for each *Gln* gene as already confirmed for SALK_148604 (*gln1;3a*) and SALK_086579 (*gln1;5*) by Dragićević et al. (2014), SALK_148604 (*gln1;3a*) and SALK_000459 (*gln1;1*) by Guan et al. (2015) and SALK_072283 (*gln1;3b*) by Moison et al. (2018).

The SALK_117504 mutant line (*gln1;5*) was added much later to the project as first mutant line tested for the *Gln1;5* gene (SALK_107993) revealed not to be a knockout mutant. By the end of the work this new line was being genotyped.

Although the *Gln1;1* is not a preferential target of this study a mutant line was successfully genotyped (**Figure 10**) for an eventual future creation of GS double mutants, and eventually a triple mutant (*gln1;1*/*gln1;3*/*gln1;5*).

The cross fertilization between *gln1;5* (SALK_086579) and *gln1;3* (SALK_148604) mutants was performed, the seeds collected and sown (F_0), but after more than 140 plants (F_1) being genotyped from different hemizygous plants, not even one double homozygous (*gln1;3*/*gln1;5)* was found. Thus, to infer about this double mutant viability, a germination assay (**Figure 12**) was performed as defined in section **2.7.2**.

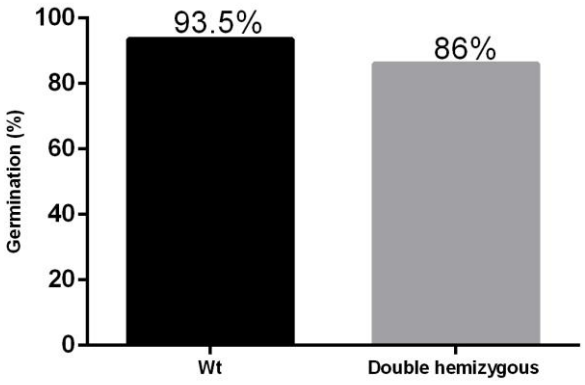


Figure 12 – Germination rate of seeds from double hemizygous (*gln1;3*/*gln1;5*) plants compared with Wt (wild-type). The seeds were placed in 5 mM nitrogen (NO_3^-) concentration medium, and the rate of germinated seeds determined 5 days after soaking.

The results of the germination assay allow us to note that seeds from the double hemizygous plants have a lower gemination rate, germinating less 7.5% than wild-type plants. These results fit in the expected percentage of a double homozygous occurrence, that according to the Mendelian inheritance would be around 6.25%.

The possibility of the double mutant unviability is in accordance to the high and specific levels of expression that *Gln1;3* and *Gln1;5* genes show during seed development (**3.1.1**). The phylogenetic proximity (**3.1.3**) between the two target genes reinforces the existence of a possible specific common role, and the requirement of, at least, one of these genes during seed development and germination processes. Nevertheless, doesn't exist any literature about this double mutant, and then would be pertinent new cross fertilizations and eventually a new germination assay to confirm these results.

3.3. Phenotype mutants' characterization

3.3.1. Germination and growth assays

After the homozygous plants' selection, the phenotype characterization of *gln1;3* and *gln1;5* SALK mutant lines was performed. In order to examine the effect of a loss of function of *Gln1;3* and *Gln1;5* genes at the initial phases of plant growth, germination (**Figure 13**) and seedlings growth assays (**Figure 14, 15, 16, 17**) were performed. The studies used different nitrate concentrations due to the relevant role of GS in nitrogen assimilation.

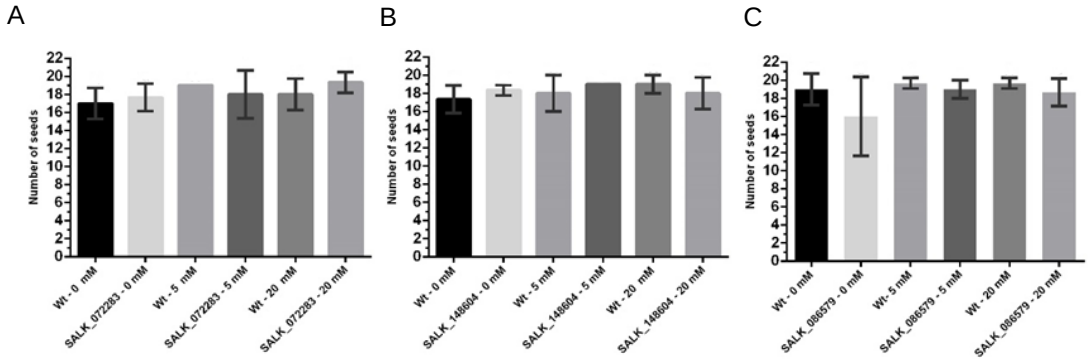


Figure 13 – Number of seeds germinated at different N conditions. The *gln1;3* (**A –** SALK_072283 and **B –** SALK_148604) and *gln1;5* (**C –** SALK_086579) and Wt (wild-type) seeds were placed in three different nitrogen concentrations (0, 5 and 20 mM of $NO3^-$) and the number of germinated seeds counted 5 days after soaking. The data are presented as the mean values +/- SD (n = 3). One-way ANOVA tests were performed, with Tukey's multiple comparison tests, ($P \leq 0.05$).

The germination assay (**Figure 13**) shows no statistical differences between the germination of wild-type seeds and also for the mutant seeds, at different N concentrations. No differences were also found between the germination of Wt and *gln1;3* (SALK_148604 and SALK_072283) or *gln1;5* (SALK_086579) seeds, in any of the tested conditions. However, we can highlight a slight tendency of *gln1;5* mutants to have a lower number of germinated seeds in all N concentrations, when compared to wild-type.

For seedling root and shoots growth assays, measures were made at the 5th, 10th and 15th day after soaking. When comparing the wild-type roots in different N concentrations, on the same measure day, significant differences are found, and a tendency of Wt roots in N excess to be shorter than at lower N concentrations are seen in most situations (**Figure 14**). For *gln1;3* mutants (SALK_072283 and SALK_148604) the plantlets at higher N concentrations tend to have longer roots (**Figure 14**) and significant results were determined for both mutant lines in most situations (**Table S1**). The *gln1;5* mutant (SALK_086579) had no differences between different N concentrations at the same day, but the roots at higher N concentrations were always shorter (**Figure 15**).

When compared with Wt plants, the results for roots length of *gln1;3* mutants showed significant differences, but not those of *gln1;5* plants (**Figure 14, 15**). On the 5th day both *gln1;3* mutants (SALK_072283 and SALK_148604) had shorter roots than Wt plants for all N concentrations, but only in the plates without nitrogen, 0 mM, the two mutants presented significant differences (**Figure 14**). At the 10th day of growth the two *gln1;3* mutants continue to have shorter roots in the lower nitrogen concentrations (0 mM and 5 mM), but these values are not significant contrasting with 20 mM nitrogen plates, which have longer roots in the mutants and these differences are significant for both *gln1;3* lines (**Figure 14**). Fifteen days after soaking the mutants' roots length in 0 mM nitrogen and 5 mM concentration continue to be shorter than Wt plantlets, but these differences are also not significant. In 20 mM plates the *gln1;3* mutant roots are longer than Wt and the differences significant (**Figure 14**). In short, these *gln1;3* plants tend to have shorter roots than Wt in absence or a normal nitrogen concentration, but longer at high nitrogen concentrations.

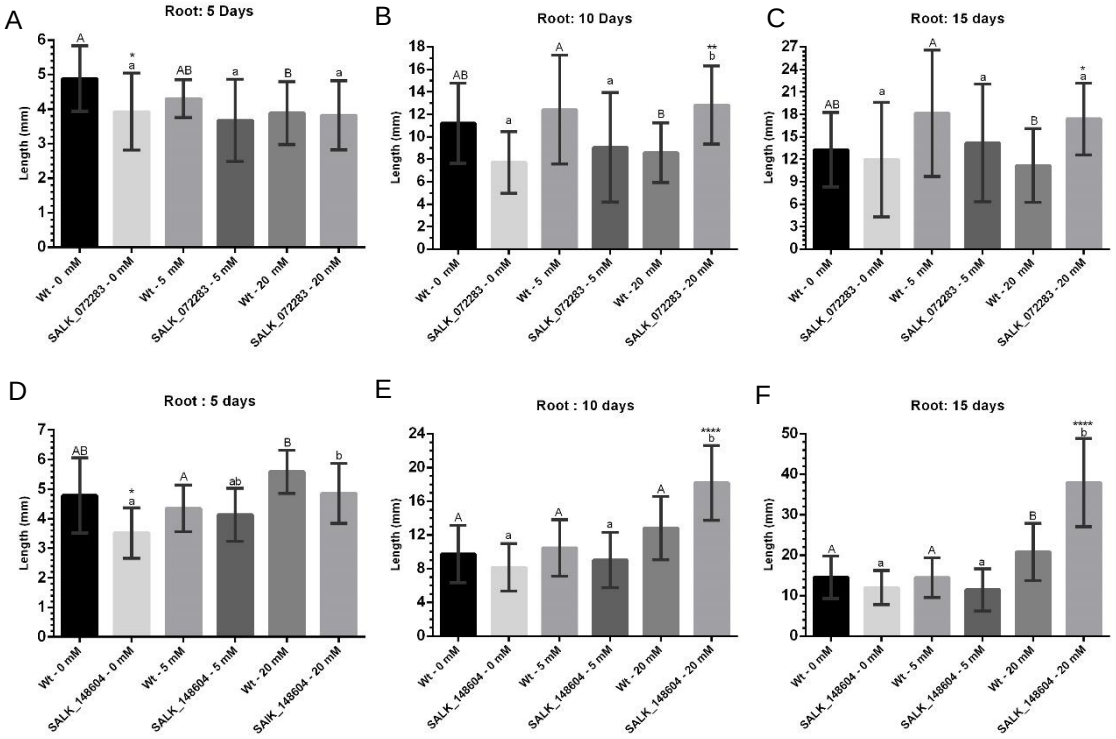


Figure 14 – Root growth under different N conditions. Measurements were made at the 5th (**A**, **D**) 10th (**B**, **E**) and 15th (**C**, **F**) day after soaking. The *gln1;3* (**A**, **B**, **C -** SALK_072283), (**D**, **E**, **F -** SALK_148604) and Wt (wild-type) seeds were germinated in three different nitrogen concentrations (0, 5 and 20 mM of NO_3^-). The data are presented as the mean values +/- SD (n = 3). One-way ANOVA tests were performed, with Tukey's multiple comparison tests. * indicates statistically significant differences between Wt and mutant plants in the same day and N condition, P ≤ 0.05. ** P ≤ 0.01. **** P ≤ 0.0001. A, B, a, b – Capital letters identify differences between Wt roots for different N concentrations and lowercase letters between the mutant roots.

The root growth assay for the *gln1;5* mutant (SALK_086579) didn't show any significant differences between wild-type and mutant roots (**Figure 15**), but overall the *gln1;5* mutant presented slightly shorter roots in all N concentrations.

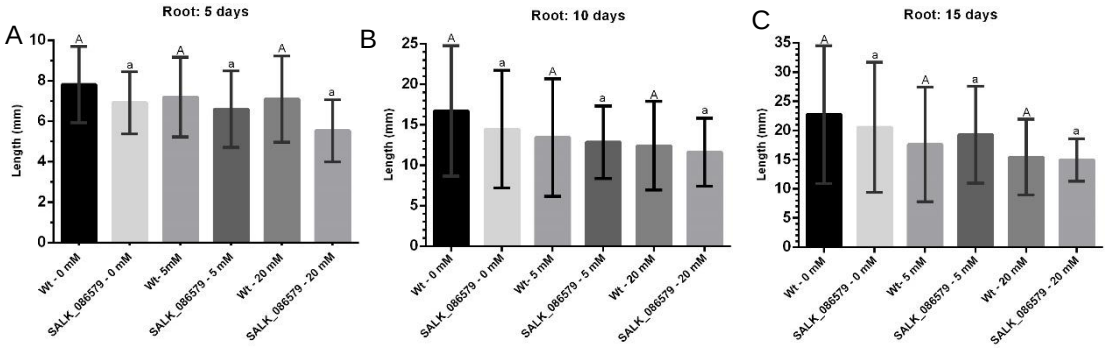


Figure 15 – Root growth under different N conditions. Measurements were made at the 5th (**A**), 10th (**B**) and 15th (**C**) day after Wt (wild-type) and *gln1;5* (**A, B, C -** SALK_086579) seeds were sown in the plates, in three different nitrogen concentrations (0, 5 and 20 mM of NO3-). The data are presented as the mean values +/- SD (n = 3). One-way ANOVA tests were performed, with Tukey's multiple comparison tests, (P ≤ 0.05). A, a – Capital letters identify differences between wild-type roots for different N concentrations and lowercase letters between the mutant roots.

As was done for roots, growth assays were also performed to measure shoots' length. The wild-type shoots (**Figure 16, 17**) at different N concentrations on the same measure day show in all assays a tendency to be longer with higher N concentrations, with significant differences (**Table S1**). For *gln1;3* (SALK_072283 and SALK148604) and *gln1;5* (SALK086579) mutants, the same tendency was verified also with significant differences, with higher N concentrations producing longer shoots (**Figure 16, 17**).

When comparing *gln1;3* shoots with wild-type (**Figure 16**), we can see that both *gln1;3* mutants have longer shoots at 5 mM and 20 mM nitrogen concentrations, by the 5th and 10th days, and in the 15th the shoots of *gln1;3* shoots become shorter than Wt. The *gln1;5* (SALK_086579) mutant shoots don't show any specific tendency in their growth, having no differences when compared with wild-type.

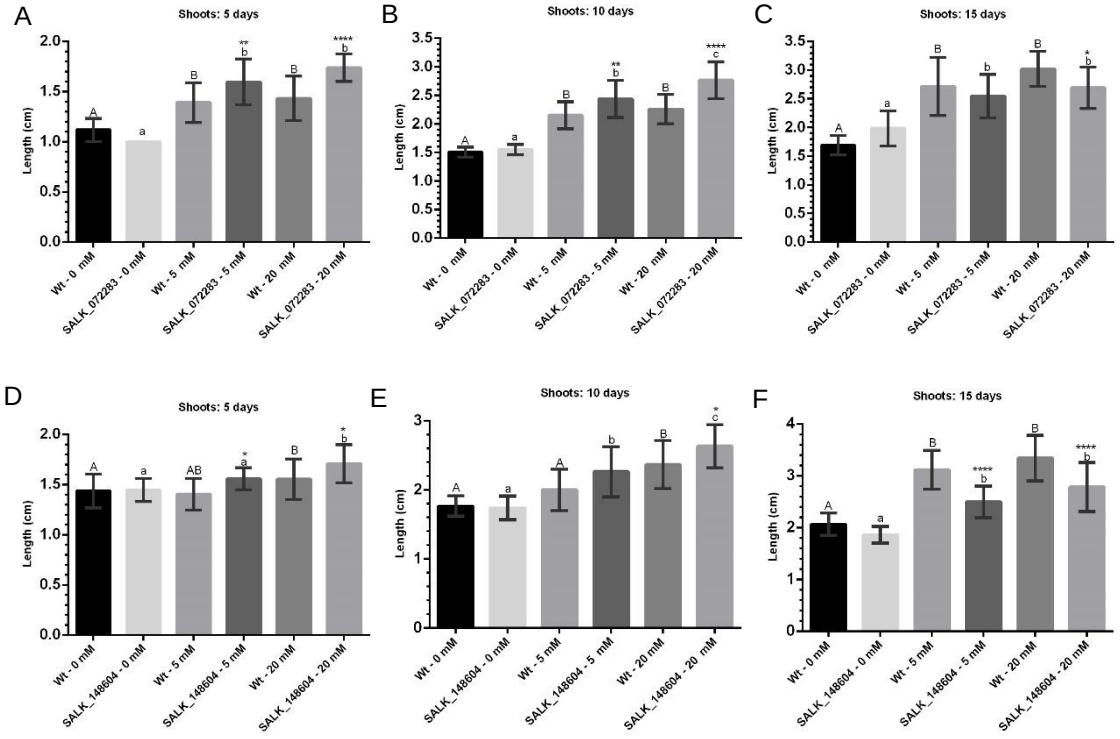


Figure 16 – Shoots growth under different N conditions. Measurements were made at the 5th (**A**, **D**), 10th (**B, E**) and 15th (**C, F**) day after Wt (wild-type) and *gln1;3* (**A, B, C -** SALK_072283 and **D, E, F -** SALK_148604) seeds were sown in the plates, in three different nitrogen concentrations (0, 5 and 20 mM of $NO3^-$). The data are presented as the mean values +/- SD (n = 3). One-way ANOVA tests were performed, with Tukey's multiple comparison tests. * indicates statistically significant differences between Wt and mutant plants in the same day and N condition. P ≤ 0.05. ** P ≤ 0.01. **** P ≤ 0.0001. A, B, a, b, c – Capital letters identify differences between wild-type shoots for different N concentrations and lowercase letters between the mutant shoots.

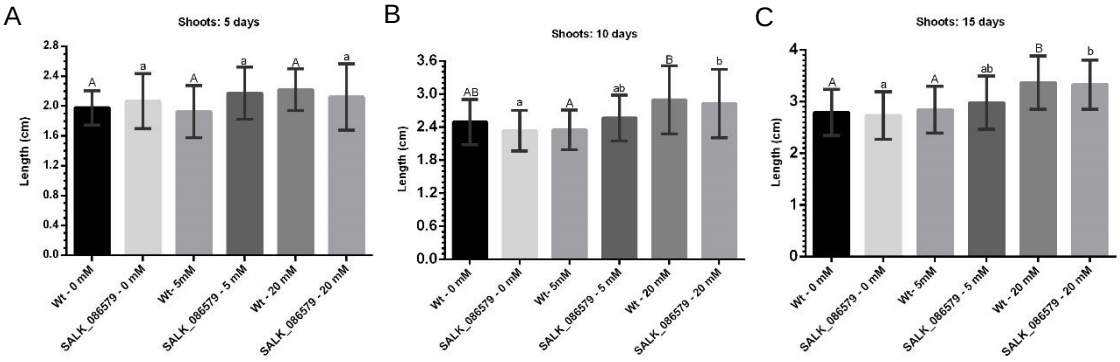


Figure 17 – Shoots growth under different N conditions. Measurements were made at the 5th (**A**), 10th (**B**) and 15th (**C**) day after Wt (wild-type) and *gln1;5* (**A, B, C -** SALK_086579) seeds were sown in the plates, in three different nitrogen concentrations (0, 5 and 20 mM of NO3-). The data are presented as the mean values +/- SD (n = 3). One-way ANOVA tests were performed, with Tukey's multiple comparison tests, (P ≤ 0.05). A, B, a, b– Capital letters identify differences between wild-type shoots for different N concentrations and lowercase letters between the mutant shoots.

After fifteen days of growth in plates, the wild-type and *gln* mutants' fresh weight was measured. The results (**Figure 18**) show that wild-type and mutant plantlets weight varied significantly (**Table S2**), having the wild-type higher mass at higher N concentrations.

Overall the *gln1;3* and *gln1;5* mutants have lower mass than wild-type plants for all nitrogen conditions, at 0 mM nitrogen concentration plates no statistical differences were detected.

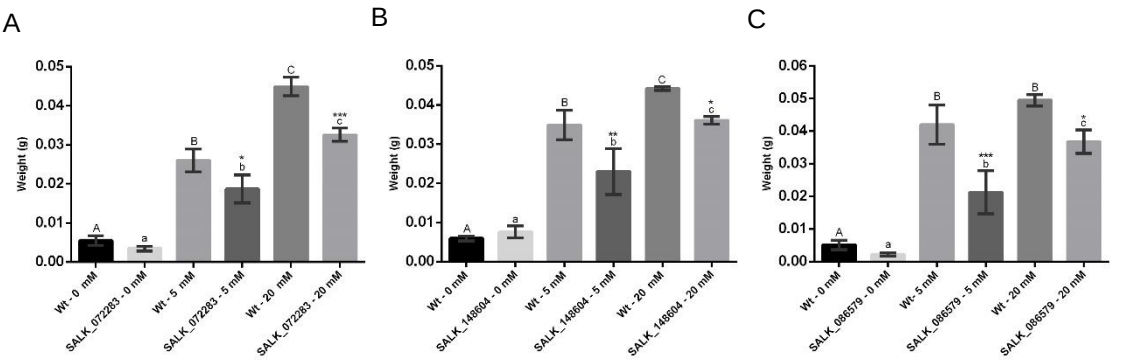


Figure 18 – Fresh weight at the 15th growth day. Fresh weight was determined in Wt (wild-type), *gln1;3* (**A –** SALK_072283 and **B –** SALK_148604) and *gln1;5* (***C* –** SALK_086579) plants in different N concentrations (0, 5 and 20 mM of NO_3^-). The data are presented as the mean values +/- SD (n = 3). One-way ANOVA tests were performed, with Tukey's multiple comparison tests, * indicates statistically significant differences between Wt and mutant plants in the same day and N condition, P ≤ 0.05. ** P ≤ 0.01 *** P ≤ 0.001. A, B, C a, b, c – Capital letters identify differences between the wild-type weight at different N concentrations and lowercase letters between the mutant plantlets.

In the assays performed in this work non-statistically significant germination results were denoted between the different N concentrations and between wild-type and mutant seeds. These results are consistent with literature to date, being so far only described that single mutant plants *gln1;1* and *gln1;2* lead to a slight delay in germination process, being the impairment reversible with exogenous N supply (Guan *et al.*, 2015).

Unlike described in Ji *et al.* (2019) the *gln1;3* mutants didn't show a consistent lower germination than Wt seeds. However, the *gln1;5* mutant had in all N conditions a lower number of germinated seeds, implicating that this gene can have some relevant function during germination process.

The root and shoots growth assays performed in this work showed, as expected, that plant growth is influenced by exterior N supply, being overall the Wt roots shorter in N excess and shoots longer with the increase of N availability (Bernard and Habash, 2009; Lea and Miflin, 2011; Betti *et al.*, 2012; Guan *et al.*, 2015). The *gln1;3* mutant plants show the same results except in what concerns the decrease in root length at high N concentration. Furthermore, compared to Wt, the roots of *gln1;3* mutants in the first 5 days of growth showed shorter length, being these differences significant in replicates with N absence. These differences can be justified by the not negligible role of *Gln1;3* in root growth in the first day after germination, though it was already verified that GLN1;3 lack leads to an increase of GLN1;1 and GLN1;2 isoenzymes, and that under low nitrogen conditions these three genes have a suggested functional redundancy (Dragićević *et al.*, 2014; Konishi *et al.*, 2018). At the 10^{th} and 15^{th} in N excess the *gln1;3* roots showed longer length than wild-type. Besides the inhibitory effect that a very high nitrate supply can have on the roots (Fredes *et al.*, 2019) and the *Gln1;3* lower expression at higher N concentrations (Ishiyama *et al.*, 2004) this could be justified by the already mentioned amount of GLN1;1 and GLN1;2 isoenzymes (Konishi *et al.*, 2018), being GLN1;1 an important enzyme in root growth (Guan *et al.*, 2015). The *gln1;3* longer roots at N excess can also be vindicated by the fact that *Gln1;2* gene can be up-regulated by external N concentrations (Lothier *et al.*, 2011; Guan *et al.*, 2016). At a 5 mM nitrogen concentration the results show that no differences were found between wild-type roots and *gln1;3* mutants, being this way the *Gln1;3* important in the first days of growth only under low N conditions. The *gln1;5* roots didn't show differences from the wild-type, confirming the already described absence of GLN1;5 enzyme at a root level (Dragićević *et al.*, 2014; Konishi *et al.*, 2017; Moison *et al.*, 2018).

The shoots growth assay showed that no differences exist between *gln1;3* and wild-type shoots length at 0 mM. So, without external N supply the *Gln1;3* gene seems to not have a fundamental role in shoot growth (contrarily to the relevant role in root growth suggested), corroborating the results by Konishi *et al*. (2018). By 5th and 10th day the *gln1;3* mutants had longer shoots, at 5 mM and 20 mM of nitrogen supply, than Wt. However, by the 15th day, in the medium with 20 mM nitrogen, the *gln1;3* mutant shoots turned out to be shorter than wild-type. In spite of the already known *Gln1;3* down-regulation by nitrate excess, it is also this enzyme the one with highest capacity for glutamine synthesis (Ishiyama *et al.*, 2004) and its activity is described in buds (Moison *et al.*, 2018). So, these results suggest that *Gln1;3* can, at this stage of development (15th day), have a determining role in the shoots when nitrogen remobilization from exterior is needed or even N detoxification. As for the roots, the *gln1;5* shoots didn't show differences in relation to the wild-type, corroborating the *Gln1;5* pollen and seed specificity (Ishiyama *et al.*, 2004; Lothier *et al.*, 2011; Dragićević *et al.*, 2014; Moison *et al.*, 2018).

The fresh weight, assay in the 15th day, showed that without external nitrogen supply the GS role will not be so demanding, and no growth differences were detected. The results in medium with N presence indicate that both isoenzymes can have activity on the external N assimilation as described for GLN1;3 in Moison *et al*, (2018), and not directly by assimilation of N from seed reserves. The mutants being lighter at 5 mM and 20 mM leads to the thought that these two isoenzymes (GLN1;3 and GLN1;5) are needed in N absorption by the root in germination/initial growth stages of the seedlings. The fresh weight assay was performed with a supply of nitrate, not contradicting the fresh weight results obtained by Konishi *et al.* (2017) for *gln1;3* mutants, in ammonium supply, where no significant differences were observed in 6 week plants.

3.3.2. Morphological characterization

To get a more detailed characterization of GS mutants used in this work, and to get more insight into the role of *Gln1;3* and *Gln1;5* GS isogenes, a visual characterization of mutants was performed, analyzing parameters like the number of leaves, flowers, siliquae and floral stems, the floral stems emergence, siliquae and floral stems length.

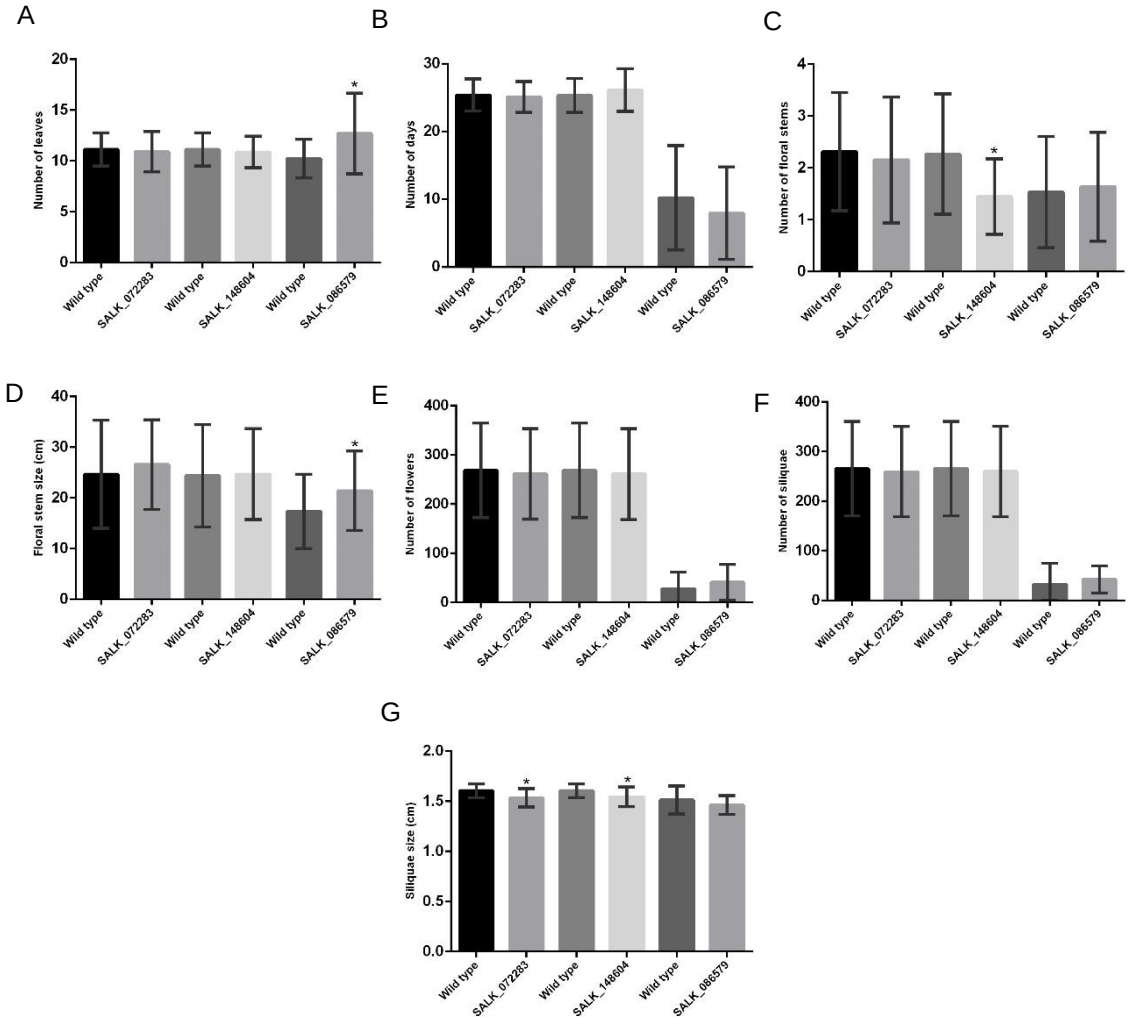


Figure 19 – Morphological characterization assays of wild-type and *gln* mutant plants. The assays analyzed the number of leaves (**A**), the number of days until the floral stem emergence (**B**), number of floral stems (**C**), floral stem size (**D**), number of flowers (**E**) and siliquae (**F**), and the siliques' size (**G**). The graphics represent the mean values in Wt (wild-type), *gln1;3* (SALK_072283 and SALK_148604) and *gln1;5* (SALK_086579) plants +/- SD (n ≥ 8). Unpaired Student's t-test were performed with Welch's correction. * indicates statistically significant differences between Wt and mutant plants in the same day and N condition, P ≤ 0.05.

None of the mutants in study reveal differences from wild-type plants concerning the number of days that floral stem lead to emerge, number of flowers or siliquae (**Figure 19B**, **19E**, **19F**). Both *gln1;3* mutants presented differences (**Table S3**) only for length of the siliquae, having shorter siliques than Wt plants (**Figure 19G**). The *gln1;5* (SALK_086579) mutant shows significant differences (**Table S3**) concerning the number of leaves (**Figure 19A**) and floral stem length (**Figure 19D**), having the mutant a slightly higher number of leaves and longer floral stems.

Until now no particular methodical assays were published comparing the growth of GS1 single mutant plants, and particularly the *Gln1;3* and *Gln1;5* mutants, with wild-type regarding the morphological parameters analyzed in this work, so there's no data available to compare the obtained results.

The *gln1;3* plants showed smaller siliquae (about 1 millimeter of difference) in accordance with *Gln1;3 in silico* results (**3.1.1**), confirming that expression in the earlier formation of the siliquae can be important to fruit development. The average number of floral stems in both *gln1;3* mutant lines was lower than wild-type plants, but only the SALK_148604 line showed statistically significant differences. These results endorse the *in silico* expression data, as *Gln1;3* has expression in meristems so its absence can possibly be related to disturbances in floral stem genesis.

The *gln1;5* plants had a slightly higher number of leaves and longer floral stems, nevertheless *Gln1;5* gene has no expression in these organs (Ishiyama *et al.*, 2004; Lothier *et al.*, 2011; Moison *et al.*, 2018) and these results (confirmed in a second assay) seem not to result from any GS expression compensation once Dragićević *et al.* (2014), show no expression alterations in the GS genes (in leaves) in *gln1;5* mutants. Overall the SALK_086579 line (*gln1;5*) seemed to produce plants more vigorous than the wild-type, thus one cannot discard the hypothesis that *Gln1;5* gene ablation can lead to some, yet, unknow interference in GS function or other close mechanisms also related, as other enzymes from nitrogen metabolism.

3.3.3. Seed set analysis

The siliquae of two months old Wt, *gln1;3* (SALK_072283 and SALK_148604) and *gln1;5* (SALK_086579) plants were analyzed, and the seed set results registered (**Figure 20**). At least twenty siliquae, in similar development stages, were chosen and four major phenotypes considered to analyze ovules and seeds: green seeds, as a product from a correct development of the endosperm and embryo; white seeds, containing embryos whose development was arrested at pre-globular stage; aborted ovules, as a result of unfertilized ovules and lastly aborted seeds, whose embryo did not maturated beyond the pre-globular stage, suffering dehydration and turning brown (additional information in http://seedgenes.org/Tutorial.html).

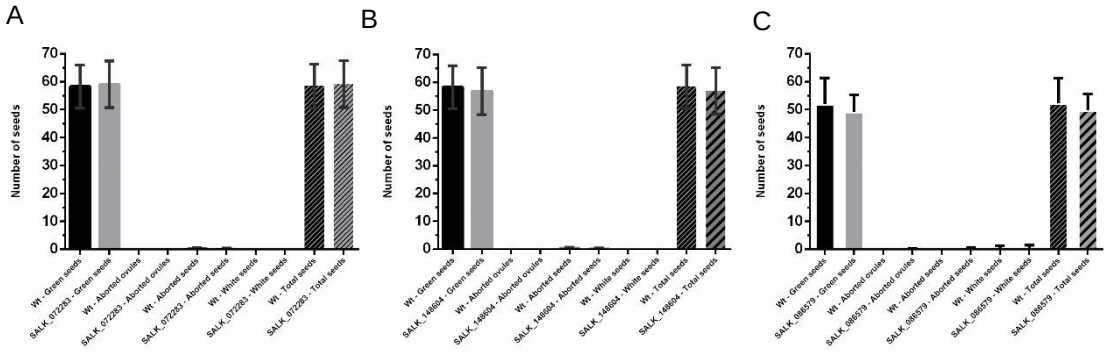


Figure 20 – Seed set production in wild-type and *gln* mutant plants. The graphics show the mean values of the seeds number for the Wt (wild-type), *gln1;3* (**A –** SALK_072283, **B –** SALK_148604) and *gln1;5* (**C –** SALK_086579) plants +/- SD (n ≥ 8). Five different parameters were evaluated in the analysis: green seeds, aborted ovules, aborted seeds, white seeds and the average number of the total number of seeds per silique was also calculated. One-way ANOVA tests were performed with Tukey's multiple comparison tests (P ≤ 0.05).

Comparing mutant lines with wild-type plants showed that none of the mutants had significant differences in any analyzed parameters. No statistical differences were also found in total number of seeds. The aborted ovules, aborted seeds and white seed parameters, had average values were near zero (**Figure 20**).

The *gln1;3* and *gln1;5* mutants results were consistent with the data obtained by Moison *et al.* (2018), determining that single mutants studied didn't present alterations in seed N remobilization or total seed yield.

3.3.4. Seed morphology assay

The mature seeds can be classified into 3 major phenotypes: round seeds, shriveled seeds and flat seeds (**Figure 21**). To identify possible anomalies in seed morphology, that could be caused by T-DNA insertions in the mutant lines, a seed morphology assessment was performed (**Figure 21**).

Figure 21 – Seed morphology. The three major seed phenotypes: **a -** Round; **b** - Shriveled; **c** - Flat.

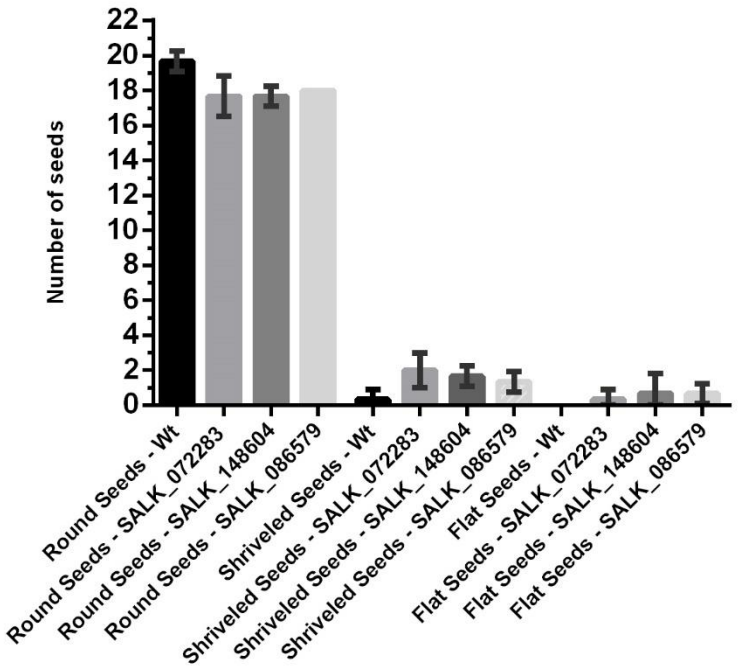


Figure 22 – Occurrence of seed phenotypes (round, shriveled and flat) in wild-type and *gln* single mutants. The graphics show the mean values of the seeds number for the Wt (wild-type), *gln1;3* (SALK_072283 and SALK_148604) and *gln1;5* (SALK_086579) plants +/- SD (n = 3). A one-way ANOVA test was performed with Tukey's multiple comparison tests ($P \leq 0.05$).

The seed morphology assay results (**Figure 22**) show that the single mutants have no statistically significant differences when compared to wild-type seeds, however the shriveled and flat phenotype have higher values of occurrence in all the single mutants. Besides the nonexistence of statistical difference, the higher number of shriveled and flat seeds in the mutants can be a consequence of the lack of each isoenzyme activity during seed formation. No other assessments of this kind are published for GS mutants in *Arabidopsis* so only with more details about the expression and location of these genes in seeds it will be possible conclude if these small differences can be related, being also more pertinent the study of a double mutant *gln1;3*/*gln1;5*.

3.3.5. In vivo pollinic tube growth assay

For further characterization of the mutants' reproductive status, reciprocal crosses between each of *gln* mutant with wild-type plants, followed by aniline blue staining of the pollen tubes, was performed. The aniline blue stains the callose walls of the pollen tubes that grow through the pistil during pollination, allowing to analyze the fertilization process. The results of this assay, that allowed the observation of pollinic tubes *in vivo,* were registered (**Figure 23, 24**). Manually were performed different reciprocal crosses for each of the mutants, essential for analyzing the existence of anomalies. One of the crosses used the Wt pistils and mutant pollen, other recurred to mutant pistils and Wt pollen. Other two type of crosses were performed and the results are in the supplements for not being essential for this analysis, Wt pistils with Wt pollen and mutant pistils with mutant pollen. The staining was performed 8 hours after pollination.

The results for *gln1;3* (SALK_148604) and *gln1;5* (SALK_086579) mutants showed that Wt pollinic tubes have grown normally in the mutant female tissues (**Figure 23A1, 24A1**) and the inverse situation revealed the same results, the mutant pollinic tubes grew normally through Wt pistils (**Figure 23A2, 24A2**). All the different crosses ended in a successful way, with Wt (**Figure 23B1, 24B1**) and mutant (**Figure 23B2, 24B2**) pollinic tubes reaching the embryonic sacs inside the eggs and without major anomalies observed.

All these crosses had no changes observed when compared to pollinic tubes normal growth in Wt plants. The pollinic tubes growth was analyzed in self-pollinated mutant plants and no anomalies were found (**Figure S4A2, S4A3**), their growth through female mutant tissues occurred without impediments, reaching the embryonic sac without alterations, the mutant pistils had also no perceptible changes or defects (**Figure S4B2, S4B3**).

These non-significant data for the *gln1;3* mutant are in agreement with the results described by Ji *et al.* (2019), that while studying the triple mutant *gln1;1*/*gln1;3*/*gln1;4* realized that single and double mutants had no fertilization anomalies, and the triple mutant, while with less mature pollen, was also viable and normal in aniline blue assessment. This 2019 research work didn't comprise the *Gln1;2* and *Gln1;5* role in the pollen. So, this confirmed viability of pollen grains in the triple mutant might foresee an important role that *Gln1;2 and Gln1;5* can have in the fertilization process.

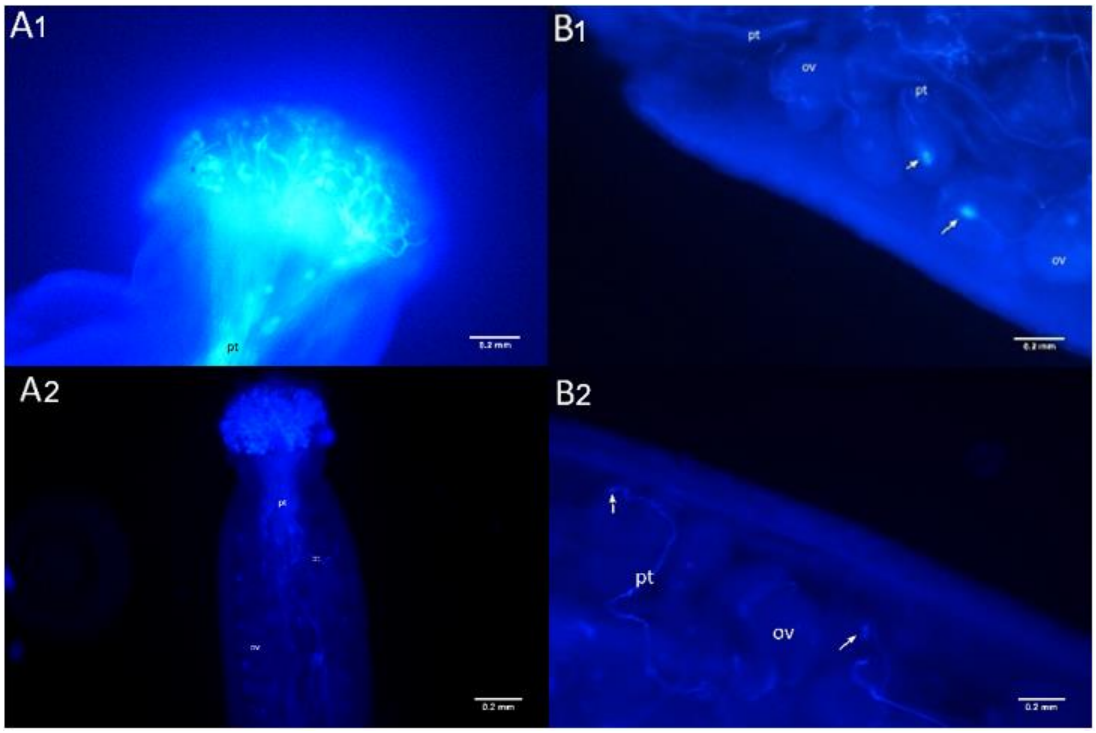


Figure 23 – Aniline blue staining after reciprocal crosses between SALK_148604 ♀ x Wt ♂ (**A1, B1**) and SALK_148604 ♂ x Wt ♀ (**A2, B2**). **A1 –** Pollen grains of wild-type plant, germinating in a *gln1;3* mutant pistil. **B1 –** Wild-type pollinic tubes growing into *gln1;3* embryo sac. **A2 –** Pollen grains of *gln1;3* plant, germinating in a wild-type pistil. **B2 –** *gln1;3* pollinic tubes growing into wild-type embryo sac.pt – pollinic tubes; ov – ovules; arrow – pollinic tubes fertilizing the ovules.

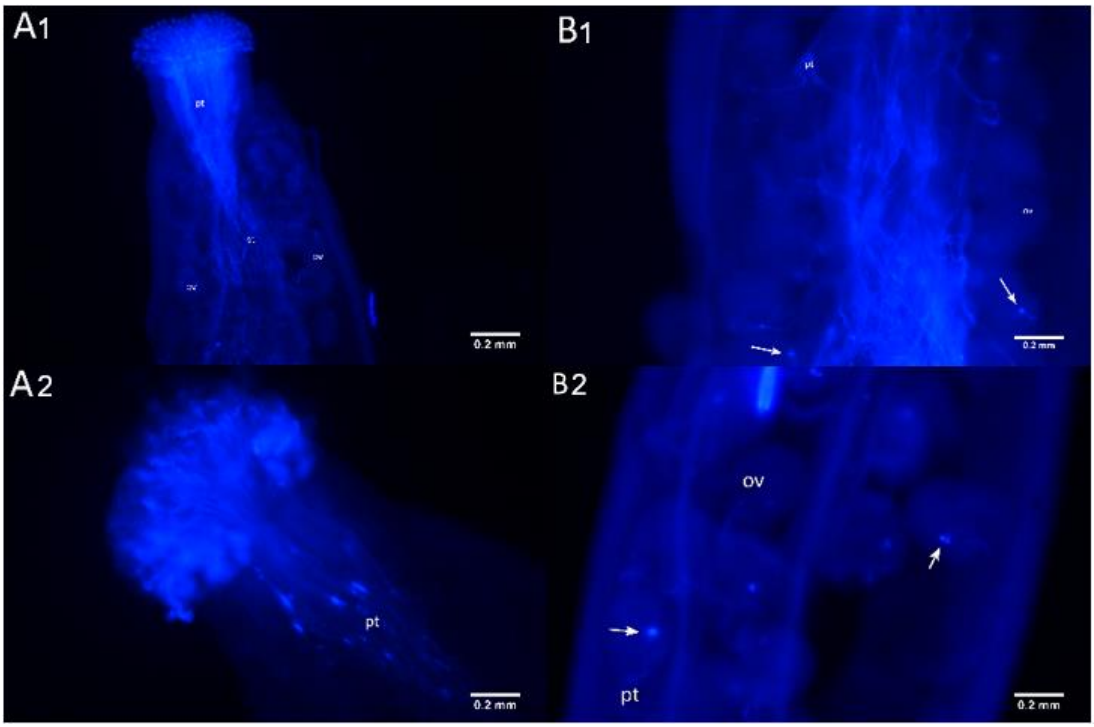


Figure 24 – Aniline blue staining after reciprocal cross between SALK_086579 ♀ x Wt ♂ (**A1, B1**) and SALK_086579 ♂ x Wt ♀ (**A2, B2**). **A1 –** Pollen grains of wild type plant, germinating in a *gln1;5* mutant pistil. **B1 –** Wild-type pollinic tubes growing into *gln1;5* embryo sac. **A2 –** Pollen grains of *gln1;5* plant, germinating in a wild-type pistil. **B2 –** *gln1;5* pollinic tubes growing into wild-type embryo sac. pt – pollinic tubes; ov – ovules; arrow – pollinic tubes fertilizing the ovules.

3.3.6. Differential staining of aborted and non-aborted pollen

Alexander's stain is a reliable and simple way to score the pollen viability. The assay permits to distinguish the grains that are viable, with a purple stain, from the pollen grains that are dead, that will stain in a soft turquoise blue (Alexander, 1969).

The pollen grains general appearance of Wt and mutant plants was registered by microscopic observation (**Figure 25**) and the grain counting was performed and the data collected organized in **Table 10**.

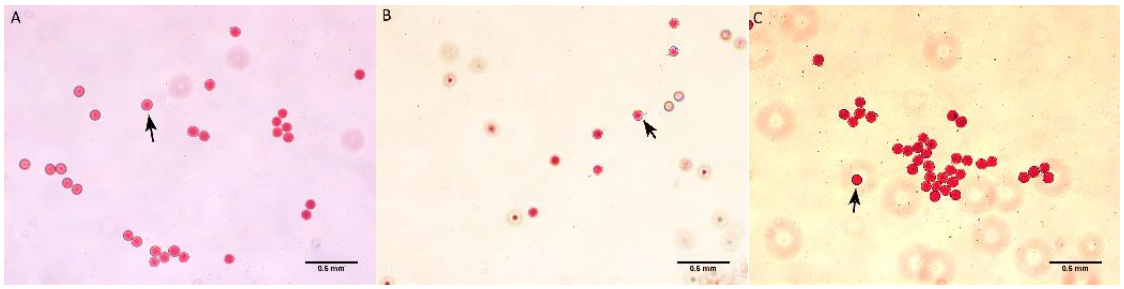


Figure 25 – Alexander staining of wild-type and *gln* mutant pollen. **A –** Wild-type; **B -** *gln1;3* (SALK_148604); **C -** *gln1;5* (SALK_086579); arrow – viable pollen grain.

The Alexander's staining results revealed that mutant pollen grains appearance was similar to the Wt (**Figure 25**), and the counting performed for all the replicates revealed no significant statistical differences in pollen viability (**Table 10**). No data is available to compare results of the Alexander dye except the work of Ji *et al.* (2019), where the staining was performed in the triple mutant *gln1;1*/*gln1;3*/*gln1;4* and anomalies in pollen maturation process were found. For *gln1;3* and *gln1;5* single mutants the lack of only one of these GS1 genes was not sufficient to compromise the pollen formation.

Table 10 – Number of viable and aborted pollen grains.

Plants	Viable Pollen	Aborted Pollen
Wild-type	600	0
SALK_148604 *(gln1;3)*	600	0
SALK_086579 *(gln1;5)*	600	0

3.3.7. Pollen germination *in vitro*

A germination assay was another approach performed to characterize the mutant pollen. This assay allows to determine if the mutant pollen had germination discrepancies from the Wt. The grains were germinated *in vitro* and analyzed after 2, 4 and 6 hours after germination. The germination was observed (**Figure 26**) and the number of germinated cells results, after 4 and 6 hours, registered (**Figure 27**). From the observations performed we can see that no differences were found between mutant and Wt germinated cells.

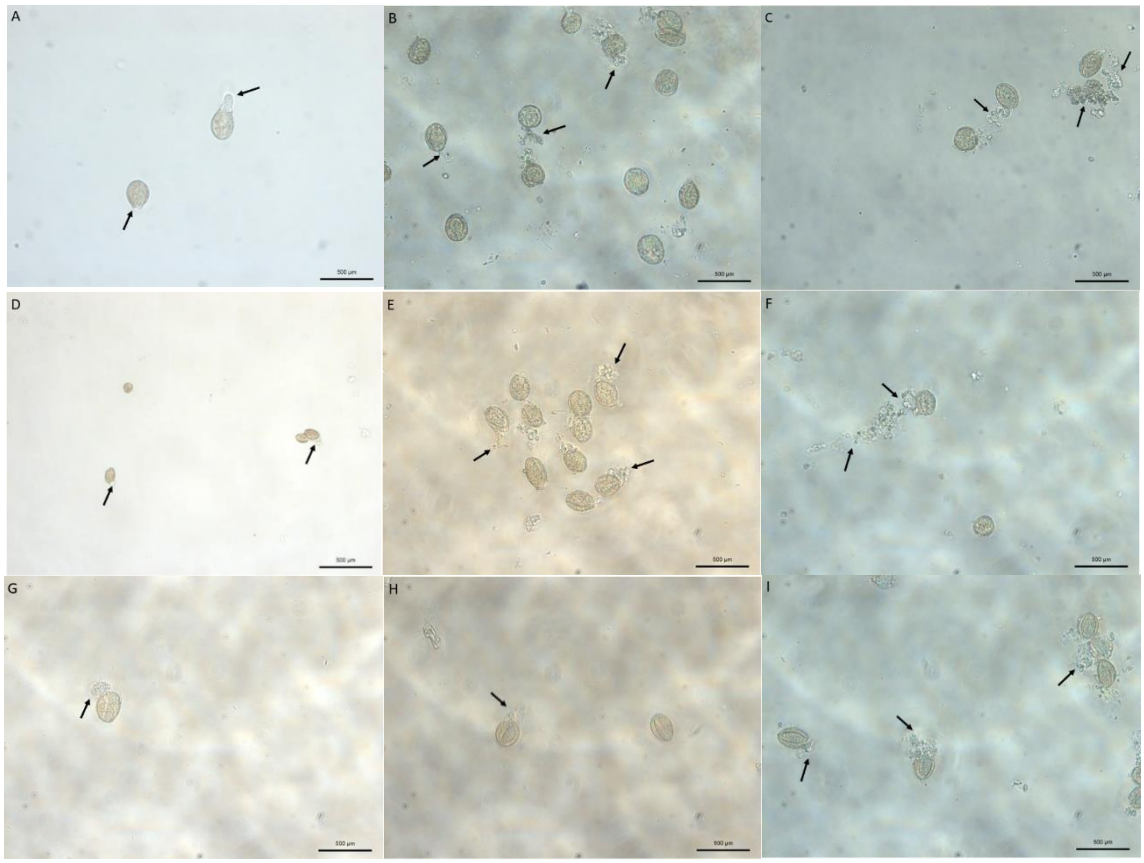


Figure 26 – Pollen grains germination assay. The pollen grains of the mutant and wild-type plants were counted at 4 and 6 hours after germination. **A, B, C –** wild-type; **D, E, F -** *gln1;3* (SALK_148604); **G, H, I -** *gln1;5* (SALK_086579); **A, D, G –** 2 hours after germination; **B, E, H –** 4 hours after gemination; **C, F, I –** 6 hours after germination; arrow – germinated pollen grain.

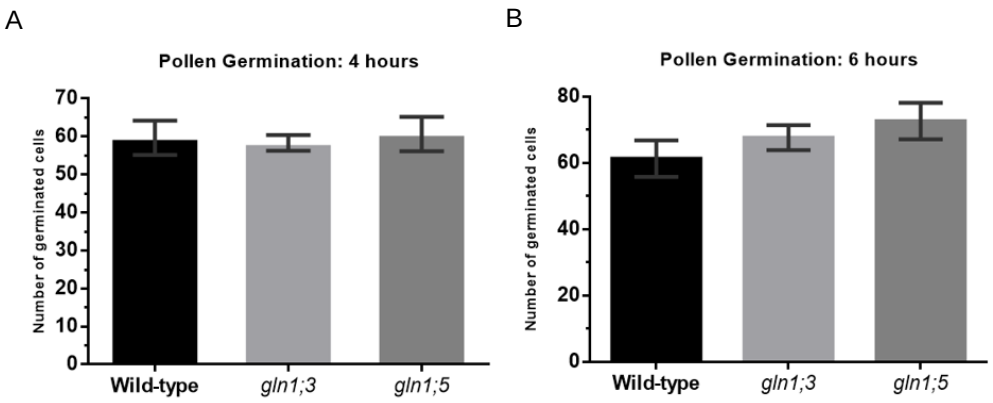


Figure 27 – Pollen germination analysis. The assay compared the pollen of Wt (wild-type) and mutant plants (*gln1;3* – SALK148604; *gln1;5* – SALK_086579), 4 (**A**) and 6 hours (**B**) after germination. The graphics show the mean values of the germinated pollen grains +/- SD (n = 3) in a total of 100 grains per replica. One-way ANOVA tests were performed with Tukey's multiple comparison tests (P ≤ 0.05).

This last assay performed with pollen grains allowed to verify that neither *Gln1;3* and *Gln1;5* genes absence lead to pollen unviability, though *gln1;5* mutant showed a higher number of germinated cells through time, this fact could be a consequence of some compensation mechanism active to suppress the lack of the *Gln1;5* in the pollen tube growth process.

3.4. Expression analysis of GS1 genes in *A. thaliana*

3.4.1. Production of transgenic reporter lines

To study the expression pattern of *Gln1;3* and *Gln1;5* transgenic marker line plants, with GUS activity conducted by their promoters, were produced. The wild-type plants were transformed by floral dip method, using *A. tumefaciens* bearing recombinant plasmids. The recombinant plasmids were obtained by using the Gateway technology and during the process the transformed entry vector (pDONR™207) was verified by enzymatic digestion using EcoRV enzyme and a sequencing analysis. The final expression vector (the pBGWFS7,0 transformed plasmid) was verified by an enzymatic digestion assay using SmaI enzyme. The electrophoresis analysis of the entry vector enzymatic digestion showed the fragments with expected molecular sizes, the non-transformed plasmid with an approximated 5585 bp fragment, the digestions with the *Gln1;3* and *Gln1;5* promoters transformed plasmids (**Figure S1A**, **S2A**) originated fragments with approximately 4581 bp and 4004 bp, respectively (**Figure 28A**).

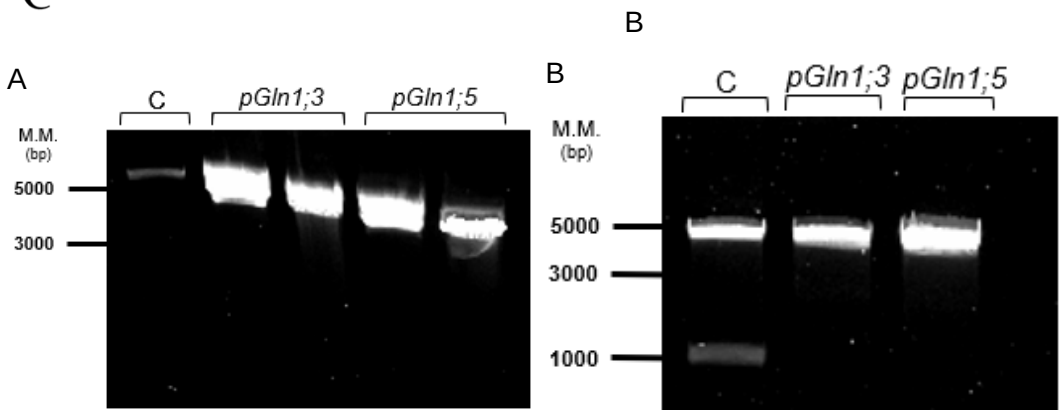


Figure 28 – Electrophoresis corresponding to the digestions performed with the transformed pDONR™207 (**A**) and pBGWFS7,0 (**B**) plasmids, for fragments verification. **C –** Original, non-transformed pDONR™207 plasmid; ***pGln1;3* –** pDONR™207 plasmid transformed with *Gln1;3* promotor; ***pGln1;5* –** pDONR™207 plasmid transformed with Gln1;5 promoter; **M.M. –** Molecular size marker.

The sequencing results (**Figure S5A-C**) evidenced the correct transformation of the entry vectors with both promoters. The final expression vectors (**Figure S1B**, **S2B**) verification results (**Figure 28B**) were the expected, the digestion performed with SmaI enzyme originates two fragments, with 11054 bp and 1397 bp approximately, in the non-transformed plasmid, and only one fragment in the transformed ones. The *Gln1;3* promoter and pBGWFS7,0 transformation has the approximated size of 12007 bp and 11430bp for the *Gln1;5*. After transformation and selection, the transgenic plants were genotyped for the gene reporter *GUS*, generating the expected 600 bp fragment (**Figure S6**), and then we proceeded with expression analysis.

3.4.1.1. Expression patterns in *A. thaliana* flowers

The putative expression of *Gln1;3* promoter exhibited GUS staining in anthers with mature pollen grains (**Figure 29A**), and when observing inflorescences, the staining is more intense in mature flowers. The early ones have a minor expression and demarcated essentially in the style and ovary walls. The flower's stigmas maintain a stronger mark through the flower maturation (**Figure 29B**). Some soft expression of the *Gln1;3* promoter is also found in petals and sepals (**Figure 29C**). The pedicel of the flowers is stained, but the receptacle region never showed any kind of coloring (**Figure 29C**). The pistil walls have strong staining in mature flowers, and the ovules show some soft coloration that gets stronger in the micropyle zone (**Figure 29D**).

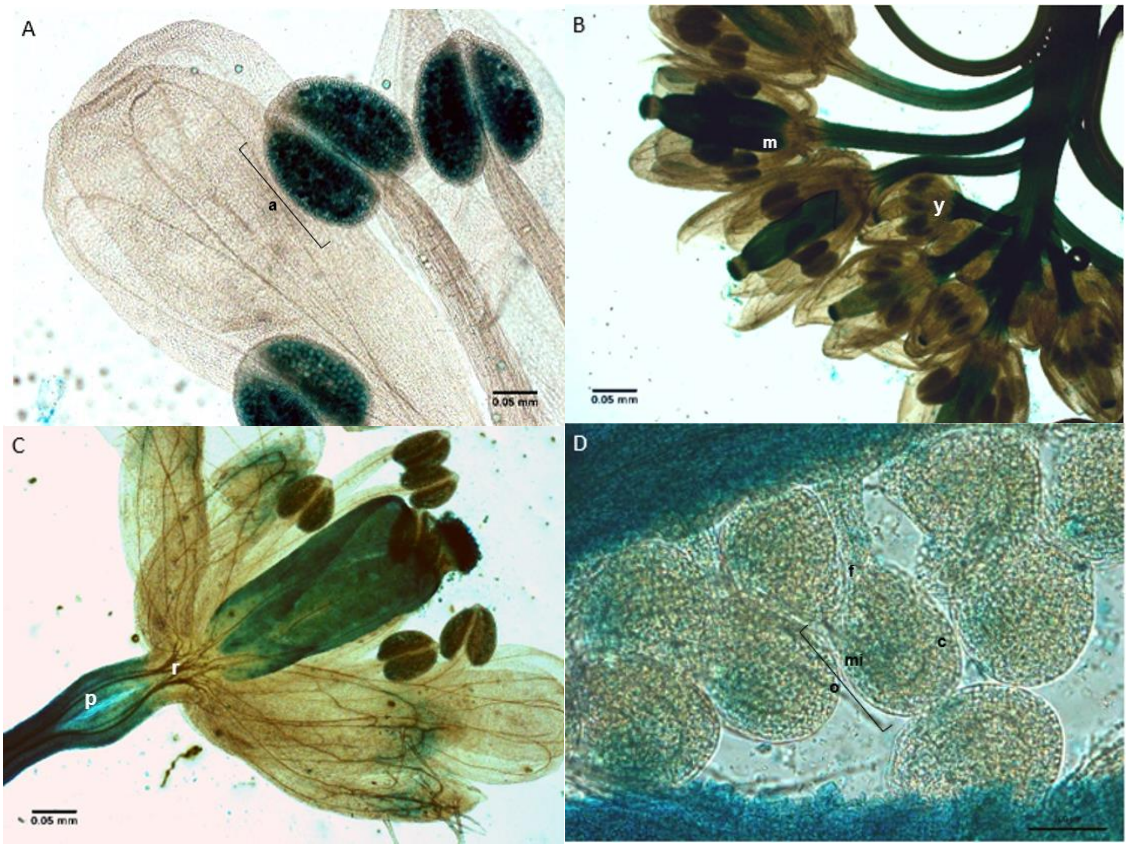


Figure 29 – Putative gene expression of *Gln1;3* in *Arabidopsis thaliana* inflorescences. Col-0 wild-type plants were transformed using *A. tumefaciens* containing p*Gln1;3*:*GUS* construct, and GUS staining assays were performed. **A –** Anthers with mature pollen grains; **B –** *A. thaliana* inflorescences; **C –** *A. thaliana* mature flower. **D –** Carpel walls and ovules. a – anther; c – chalazal pole; f – funicle; m – older flower; mi – micropyle; y – younger flower; p – pedicel; r – receptacle; o – ovule.

The flowers of trangenic mutant line with the *GUS* activity oriented by the *Gln1;5* promoter, showed that expression occurs in anthers with mature pollen grains (**Figure 30A**) and the pollinated flowers have observable staining in pollinic tubes growing through the pistil, the ovules don't have any type of mark before fertilization (**Figure 30B**). The inflorescences have a more intense staining in mature flowers, the early ones don't present any marks (**Figure 30C**). The staining always appears only in the pollen grains of the flowers, being stronger in older ones (**Figure 30D**). Twelve hours after pistil pollination the pollen grains have no longer staining, and the pollinic tubes are no more visible (**Figure 30E**), the fertilized ovules become marked, showing coloration in the micropyle area (**Figure 30F**).

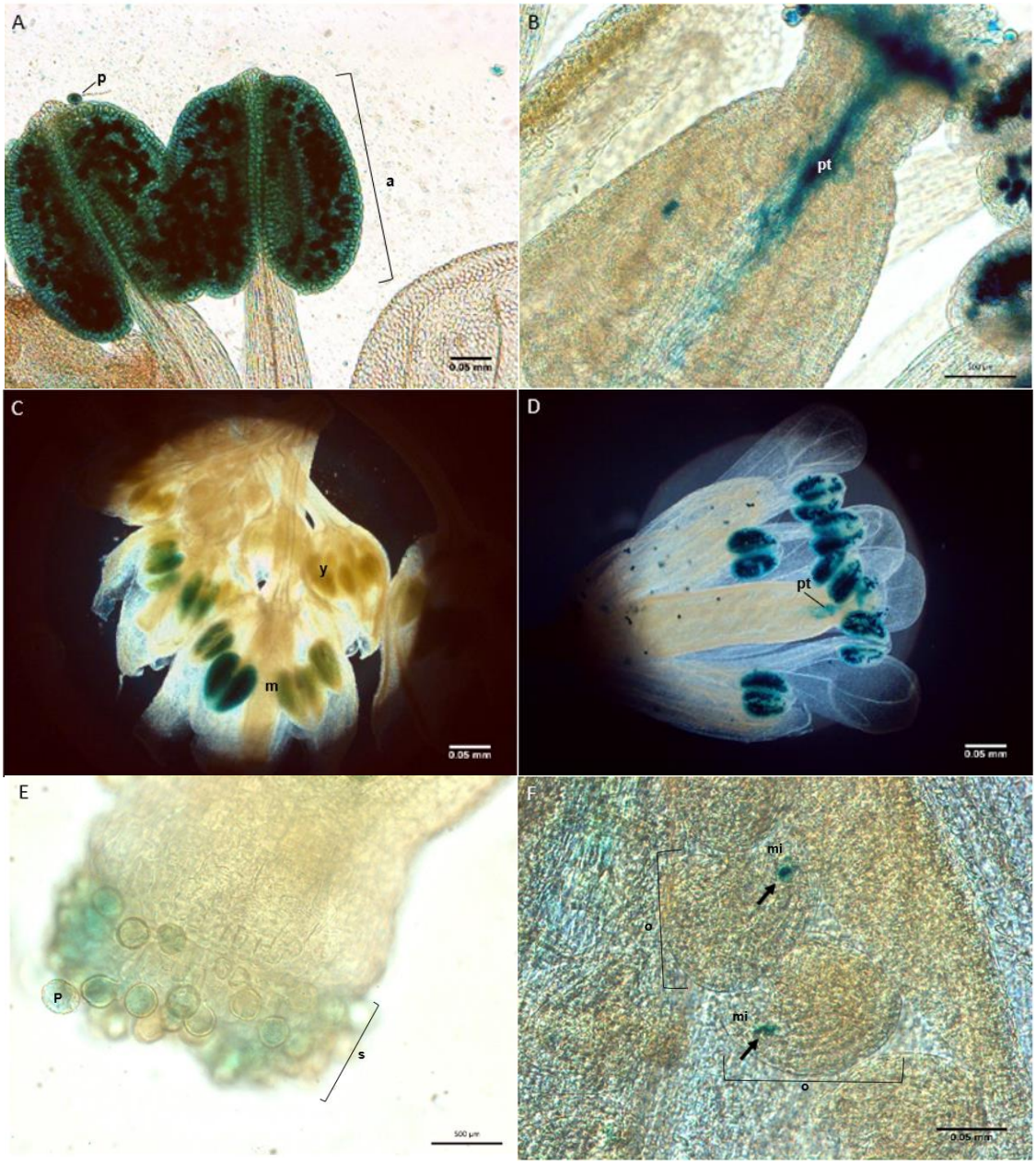


Figure 30 – Putative gene expression of *Gln1;5* in *Arabidopsis thaliana* inflorescences. Col-0 wild-type plants were transformed using *A. tumefaciens* containing p*Gln1;5*:*GUS* construct, and GUS staining assays were performed. **A –** Anthers with mature pollen grains; **B –** Pollinated flower with pollinic tubes growing through the flower's pistil; **C –** Inflorescence with pGLN1;5 staining **D –** Mature flower. **E –** Stigma with pollen, 12 hours after pollination. **F –** Fertilized ovules, 12 hours after pollination. a – anther; m – older flower; mi – micropyle; p – pollen; pt – pollinic tubes; s – stigma; y – younger flower; arrow – GUS staining;

Both promoters' expression in flowers have only pollen as a common location. The *Gln1;3* promoter is far widely expressed in flower tissues, with higher expression in stigma, like forecasted in **3.1.1**, having a role in female and male organs. In turn *Gln1;5*, seems to be a pollen specific GS, confirming so the *in silico* forecasts (**3.1.1**) and works like Dragićević *et al.* (2014) and Moison *et al.* (2018).

3.4.1.2. Expression patterns in *A. thaliana* seeds

Analysing the *Gln1;3* promoter expression in seeds with embryos on globular stage, we can see the expression is homogeneous and wide throughout all the seed tissues (**Figure 31A**). On the seeds with heart shape embryos (**Figure 31B**) the expression isn't so widespread, being stronger and almost limited to the side of the seed near the embryo (**Figure 31A**).

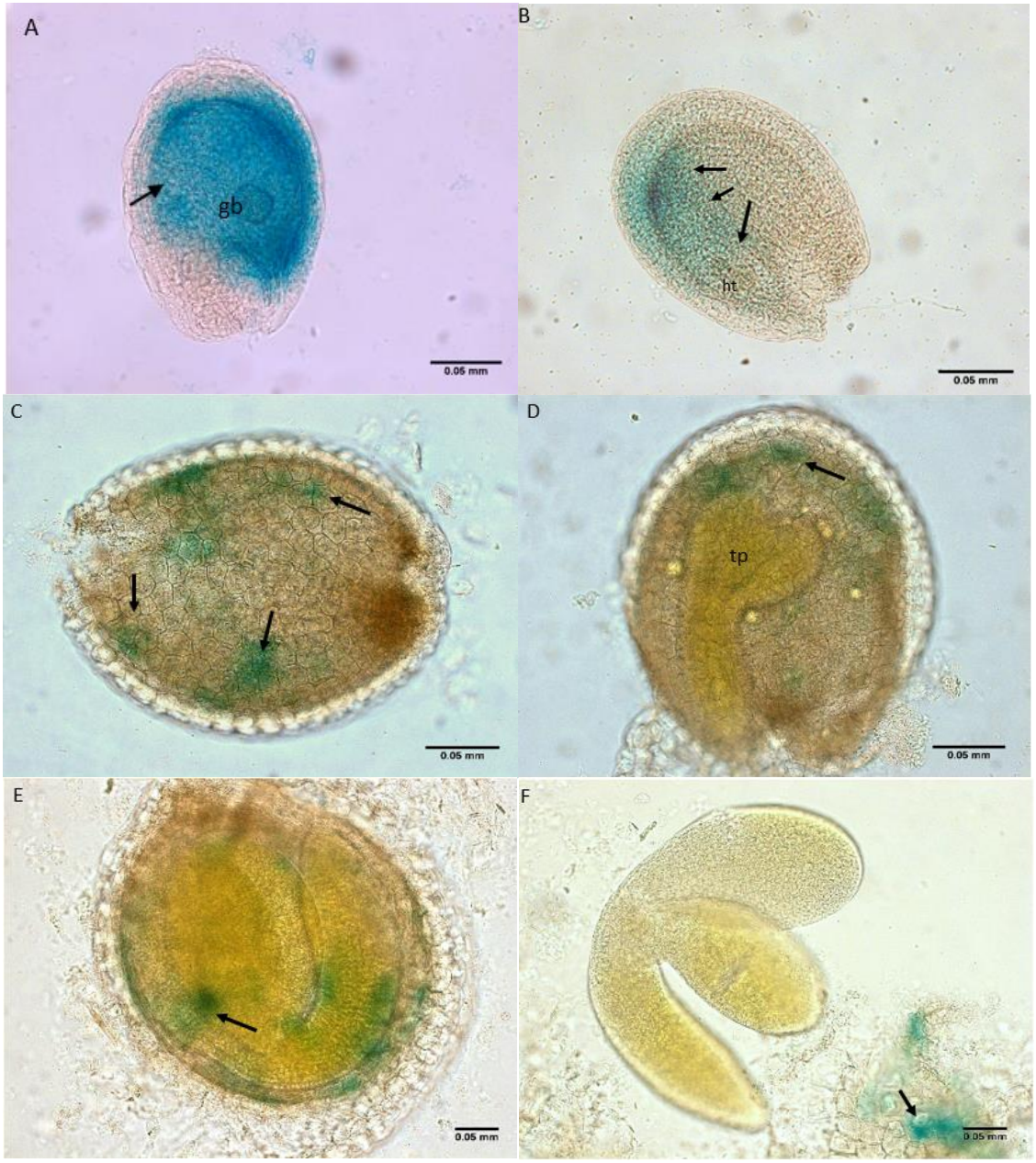


Figure 31 – Putative gene expression of *Gln1;3* in *Arabidopsis thaliana* seeds. Col-0 wild-type plants were transformed using *A. tumefaciens* containing p*Gln1;3:GUS*, and GUS staining assays were performed. **A –** Seed with the embryo in globular stage; **B –** Seed with embryo in heart shape; **C, D –** Seed with an early torpedo embryo; **E –** Seed with later torpedo embryo; **F –** Later torpedo embryo; gb – globular embryo; ht – heart shape embryo; tp – torpedo embryo; arrow – GUS staining.

In seeds having an early torpedo embryo the GUS staining is disperse, appearing as focus scattered throughout the seed (**Figure 31C, 31D**). The early torpedo embryos don't show any staining, the ativity is localized only on the seed tissues (**Figure 31D**). The full developed seed, but still green, with later torpedo embryos, show also *GUS* activity in isolated stain coloring (**Figure 31E**), being absent in the embryos tissues (**Figure 31F**).

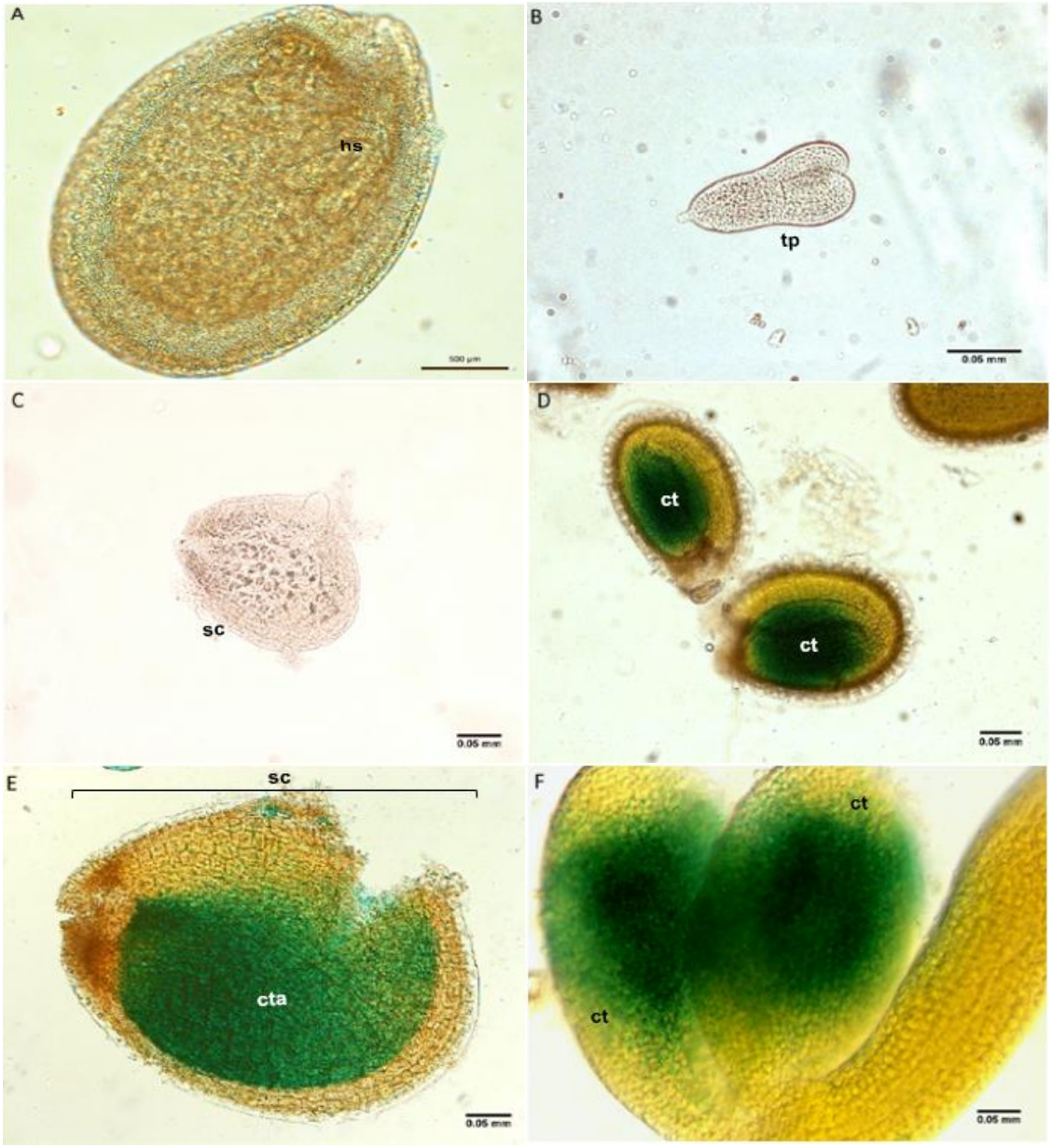


Figure 32 – Putative gene expression of *Gln1;5* in *Arabidopsis thaliana* seeds. Col-0 wild-type plants were transformed using *A. tumefaciens* containing p*Gln1;5*:*GUS*, and GUS staining assays were performed. **A –** Seed with embryo in a heart shape stage. **B –** Early torpedo embryo; **C –** Seeds coat of an early torpedo embryo; **D, E, F –** Green seeds with later torpedo embryos. ct – cotyledon; cta – cotyledons area; ht – heart shape embryo; sc – seed coat; tp – torpedo embryo;

The *Gln1;5* promoter expression is not found in seeds with embryos at early stages of development. In globular, heart shape (**Figure 32A**) and early torpedo embryos (**Figure 32B, 32C**) staining is not detected in either embryos or seed tissues.

Seeds with later torpedo embryos show GUS staining only in the cotyledons' area (**Figure 32D**). The stain occurs in seed tissues envolving the zone and in the embryo's cotyledons (**Figure 32E, 32F**).

These observations confirm that both GS1 genes studied have different expression patterns throughout seed development. The *Gln1;3* seems to have earlier activity, appearing in seed with embryos at globular stage, expressing aparently only in seed tissues, without embryo activity. The *Gln1;5* appears only in seeds with late torpedo embryos, expressing in seed and embryo tissues, at cotyledons level. The obtained results are consistent with the *in silico* data (**3.1.1**).

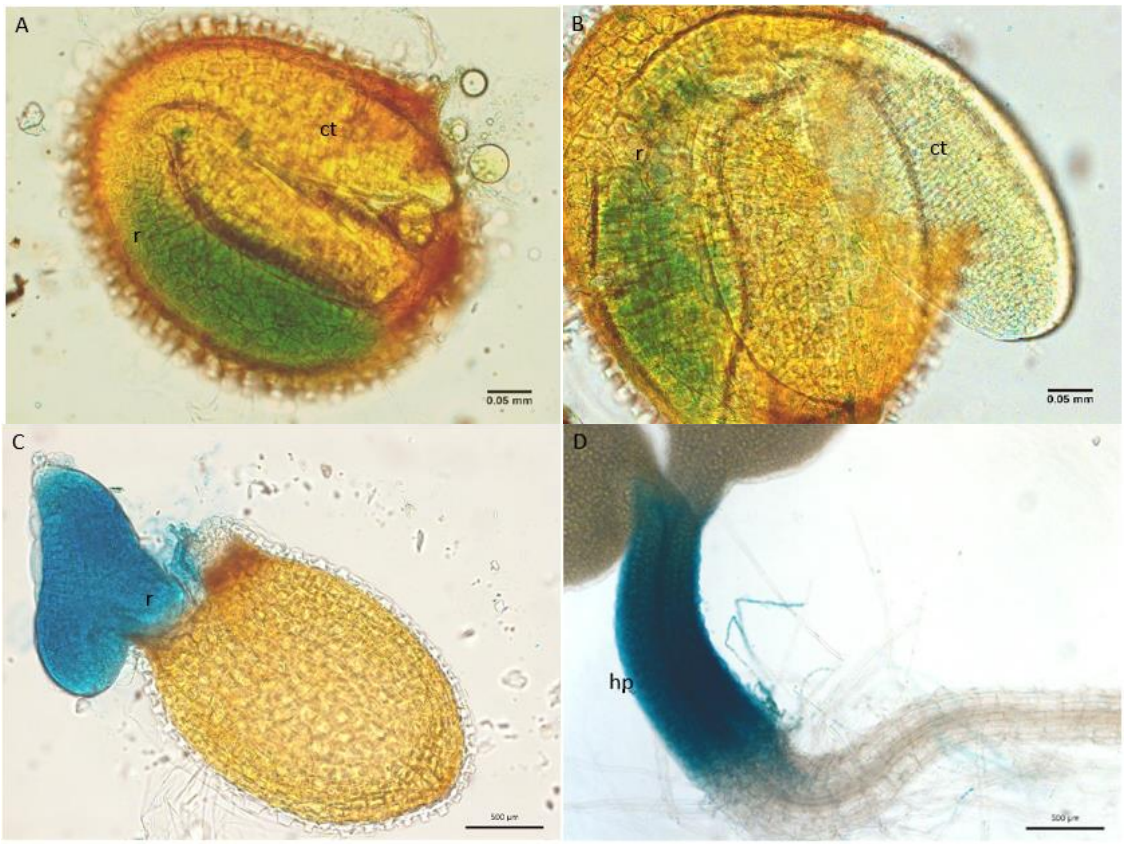


Figure 33 – Putative gene expression of *Gln1;3* in *Arabidopsis thaliana* mature and germinating seeds. Col-0 wild-type plants were transformed using *A. tumefaciens* containing p*Gln1;3*:*GUS* construct, and GUS staining assays were performed. **A, B –** Mature seed; **C –** Seed 24 hours after germination; **D –** Plantlet 72 hours after germination; ct – cotyledon; r – radicle; hp – hypocotyl.

In mature seeds the *Gln1;3* putative expression is no longer visible in the seed tissues and starts being restrict to the embryo, exclusively at radicle level (**Figure 33A, 33B**). Twenty-four hours after germination, the staining is still limited to the radicles (**Figure**

33C), and 72 hours after germination the activity decreases in the radicle and occurs mainly in the hypocotyl (**Figure 33D**).

The *Gln1;5* promoter activity in mature seeds shows GUS marking at the interior of the seed tissues and the embryo (**Figure 34A**). The staining in mature embryos occurs in all tissues, but is stronger in cotyledons and get tenuous in the radicle apex (**Figure 34B**).

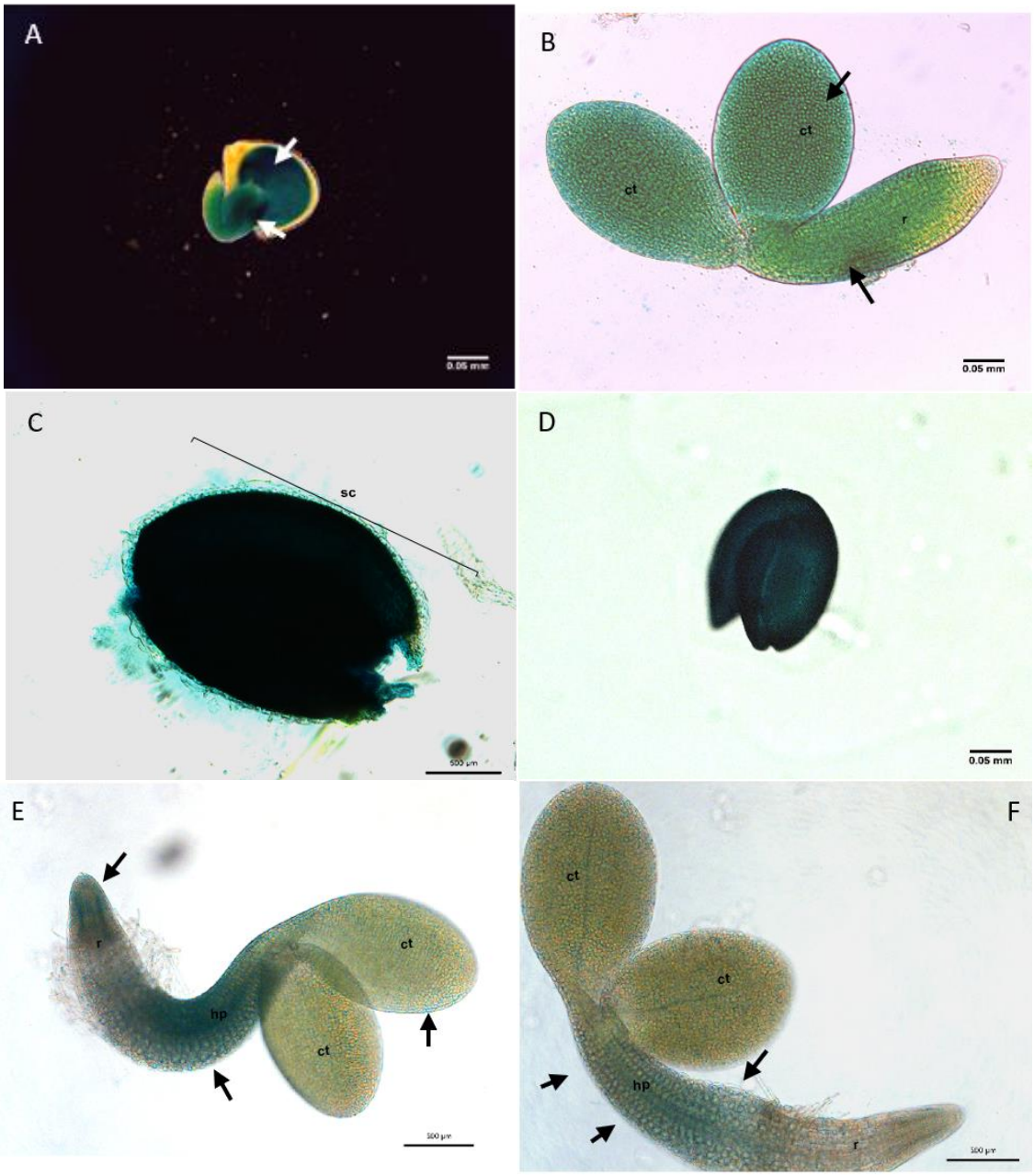


Figure 34 – Putative gene expression of *Gln1;5* in *Arabidopsis thaliana* mature and germinating seeds. Col-0 wild-type plants were transformed using *A. tumefaciens* containing p*Gln1;5*:*GUS*, and GUS staining assays were performed. **A –** Mature seed; **B –** Mature embryo; **C, D –** Seed 24 hours after germination; **E, F –** Plantlets 72 hours after germination. ct – cotyledon; hp – hypocotyl; r – radicle; sc – seed coat; arrow - GUS staining.

Twenty-four hours after germination we can see that all the seed tissues (**Figure 34C**) and embryo (**Figure 34D**) are completely marked with a stronger dark blue color. Seventy-two hours after germination the seedlings show soft marking. In smaller seedlings (**Figure 34E**) the radicle is still full stained and in seedlings with the same time of growth but more developed the stain disappears from the extremities of the tissues being visible as a soft light coloration in hypocotyl zone (**Figure 34F**).

The genes studied, *Gln1;3* and *Gln1;5* show also in mature seeds and at 24 hours after germination different patterns of expression. While *Gln1;3* expression is restricted to radicle, the *Gln1;5* has a wider localization throughout the seed. At 72 hours after germination both genes appear to have a common expression, restricted to the hypocotyl tissues, and much more fainted. Unlike what happened in flowers, the *Gln1;3* promoter in seeds has a less spread expression, restricted to the seed coat in the first stages of development and later to the radicle, and *Gln1;5* a wider activity, reaching the whole seed tissues and embryo.

The *Gln1;3* and *Gln1;5* expression patterns obtained coincide with the *in silico* research (**3.1.1**). The way both genes express during maturation phases of the seed, and taking in fact their evolutionary proximity (**3.1.3**), a possible combined activity in the nitrogen remobilization can exist. *Gln1;3* expresses in initial stages of seed development at seed coat level, while there is no expression of the *Gln1;5* gene. Later, the *Gln1;3* promoter activity fades away from the seed coat while *Gln1;5* is by now expressing, remobilizing nitrogen at cotyledons level. In the mature seeds the *Gln1;3* starts expressing in the embryo with radicle specificity and the *Gln1;5* promoter activity spreads from the cotyledons to all embryo's body, but less strong in the radicle, existing possibly a complementary effect. By the initial hours of germination, both genes still present common expression in radicle, where growth is more active at this stage of development and later on, at 72 hours after imbibition, end up being confined at hypocotyl area, a source organ in nitrogen remobilization (Hong *et al.*, 2012; Guan *et al.*, 2015).

This sequence of expression fits with the likely GS role in embryo's development, where the ammonium produced is initially remobilized from cotyledons (source organs) to developing sinks (Guan et al., 2015), this way the *Gln1;5* gene is an undeniable piece of this mechanism. The *Gln1;3* is earlier oriented to the ammonium kidnapping from seed coat, and later expresses in embryos at radicle level, confirming its role in roots (Ishiyama *et al.,* 2004, Konishi *et al.*, 2017 and Moison *et al.,* 2018).

3.4.2. RT-qPCR

3.4.2.1. Wild-type study

One of the main aims of this work was to study the expression of GS1 genes in the reproductive process in *A. thaliana* and to assess the importance of certain GS1 genes in this mechanism. In a first approach the analysis of GS1 gene expression in wild-type plants, by RT-qPCR, was performed. This analysis allowed a better comprehension of the GS1 genes different expression patterns during seed formation (only studied in transcriptome-wide expression profiling) and to pinpoint the particular seed development stages and reproductive organs that could be pertinent to scrutinize in the GS1 mutant plants. Several seed stages development, the pollen and the flowers in stage 12 of development (as defined by Smyth *et al.*, 1990), from wild-type plants were analyzed. All the GS specific primers used in the real time studies of this work were primarily submitted to efficiency tests (**Figure S7**). The *Gln1;4* gene, being a senescence specific GS and in the *in silico* analysis (**3.1.1**) absent from seed and reproductive tissues, was excluded from the real time studies.

Concerning GS1 gene expression in all flower tissues, *Gln1;2* is the gene more highly expressed in stage 12 (Smyth *et al.*, 1990) flowers (**Figure 35**), being the only with significant differences of expression relatively to other *gln* genes. *Gln1;3* is the second most expressed GS1 gene in flower, and *Gln1;1* and *Gln1;5* the genes with lower levels of transcripts.

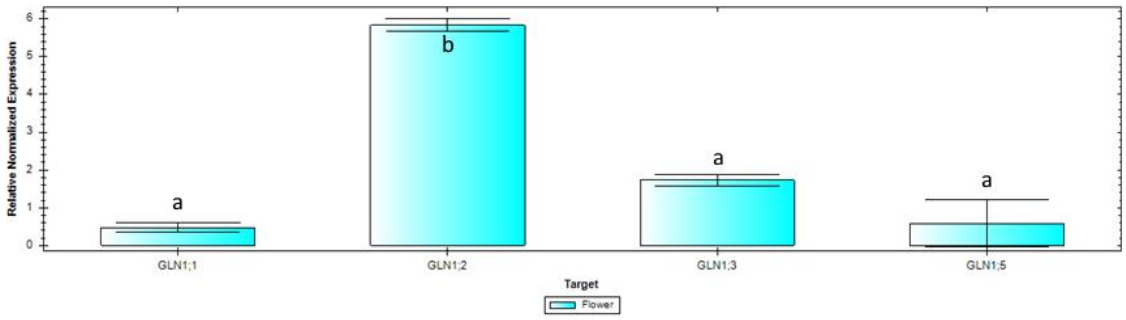


Figure 35 – Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana* wild-type in stage 12 flowers (Smyth *et al.*, 1990). Each of the bars represent the average, +/- SD (n = 3). The different letters (a, b) define significant differences (P ≤ 0.05) between the different *Gln* genes.

In the pollen of stage 15 flowers (Smyth *et al.*, 1990), the *Gln1;1* and *Gln1;2* have higher and similar expression values (**Figure 36**), and the *Gln1;5* the gene with low values.

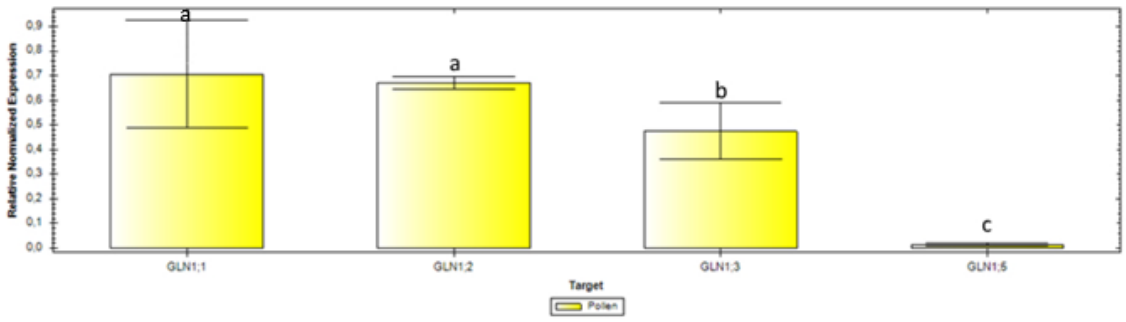


Figure 36 - Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana* wild-type pollen of stage 15 flowers (Smyth *et al*., 1990). Each bar represents the average, +/- SD (n = 3). The different letters (a, b, c) define significant differences (P ≤ 0.05) between the different *Gln* genes.

These results show that in flowers at stage 12 of development and pollen from stage 15 flowers (Smyth *et al.*, 1990) the *Gln1;5* has expression but at very low levels. The major expression of *Gln1;2* in both cases and the *Gln1;3* appears as the second most expressed in flowers and the third in pollen. These results corroborate the idea that GLN1;1, GLN1;2 and GLN1;3 are the three major GS1 isoforms in flowers/pollen as already observed by GUS histochemical detection for the *Gln1;3* gene (**Figure 30**) and pointed by other authors (Lothier *et al.*, 2011; Dragićević *et al*., 2014 and Moison *et al.*, 2018).

Concerning the expression of GS1 genes during seed formation, transcript levels were determined in four stages of seed development, seeds with heart shape embryos, with early torpedo embryos, green seeds (later torpedo embryos) and mature seeds. In each stage of seed development, the expression of the different GS1 isogenes were determined by RT-qPCR. The data figures, related to the expression in each individual stage are shown in **Figure 37**. In the heart shape stage, the *Gln1;2* is the most highly expressed gene, having the other isogenes similar expression (**Figure 37A**). In early torpedo seeds all the isogenes have similar levels of expression (**Figure 37B**). The later stages of development, in green and mature seeds, the *Gln1;5*, is the GS1 isogene with higher levels of expression (**Figure 37C, 37D**). In mature seeds (**Figure 37D**) the *Gln1;3* is the second most expressed and the *Gln1;1* has higher expression than *Gln1;2,* being this last the gene with the lowest level of transcripts.

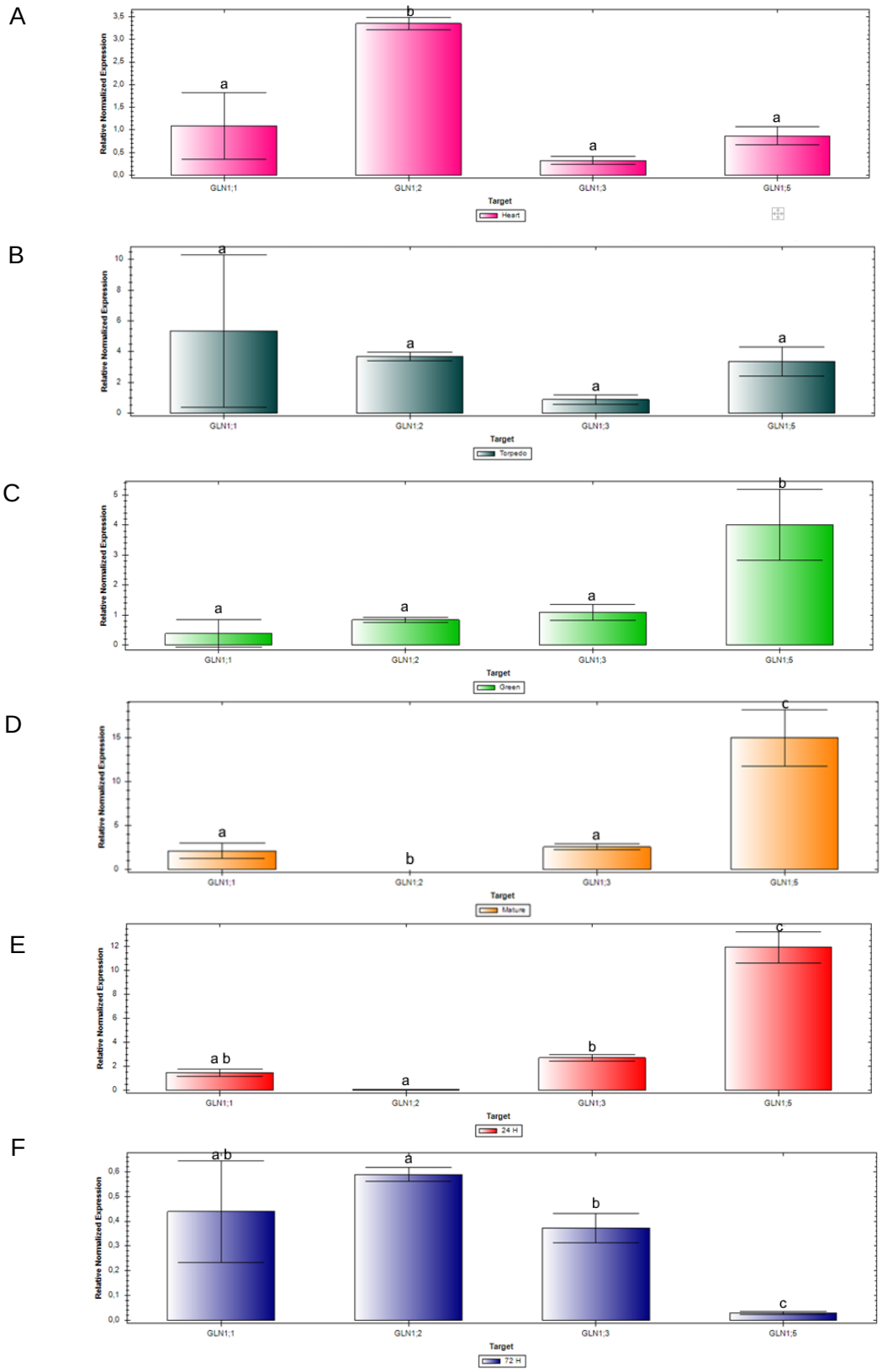


Figure 37 – Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana* wild-type plants in different seed development stages. **A -** Heart shape embryo; **B –** Early torpedo embryo; **C –** Green seeds (later torpedo embryo); **D –** Mature seeds; **E –** Seeds 24 hours after germination; **F –** Seeds 72 hours after germination. The different letters define significant differences (P ≤ 0.05) between *Gln1* genes, in the same stage of seed development.

Furthermore, we have analyzed the patterns of expression of GS1 isogenes during early phases after germination. At 24 hours after germination (**Figure37E**) the *Gln1;5* continues to have highest expression of the GS1 genes, being the *Gln1;3* the second most expressed gene and the *Gln1;2* the one with lowest values. Finally, 72 hours after germination the *Gln1;5* has the lowest expression value of the four genes, becoming the *Gln1;2* the most expressed gene (**Figure 37F**).

Over seed formation (**Figure S8**) the *Gln1;1* expression changed in a nonlinear way, increasing from the heart shape to the early torpedo embryo stage, decreasing in the green seed and increasing again the levels of expression in the mature seed. The *Gln1;2* relative expression values are maintained in the heart shape and early torpedo stages, lowering in the green seed and decreasing to an even lower values in the mature seed, pointing to a role of this isoenzyme only in the early stages of the seed formation. *The Gln1;3* and *Gln1;5* expression during seed development increases until seed maturation, having the *Gln1;5* the highest relative expression increments during the process and being, as stated before, the isogene with highest expression in mature seeds. These patterns of expression suggest a relevant role for these isogenes during seed formation, and confirms the *in silico* data (**3.1.1**), legitimating the choice of both genes as the main focus of this research.

Focusing on germination process (**Figure S9**) the wild-type expression analysis revealed that *Gln1;1*, *Gln1;3* and *Gln1;5* transcript levels decrease during germination, suggesting the reduction of importance of these genes to plantlets after germination. The *Gln1;2* is the only gene with successive increasing values during germination, changing from an almost inexistent expression in mature seeds to the most expressed in plantlets, pointing to a relevant role of this GS in *A. thaliana*, corroborating also this gene as the highly expressed in *A. thaliana grown* plants (Schmid *et al.*, 2005; Lothier *et al.*, 2011; Guan *et al.*, 2015; Guan *et al.*, 2016; Guan and Schjoerring, 2016).

The data produced by RT-qPCR analysis can be compared with the *GUS* expression assay performed (**3.4**). At the beginning of seed formation the *Gln1;3* and *Gln1;5* expression is reduced in both studies, increasing through the seed maturation process, in every phase of the visual expression analysis is possible to relate with the same phase in the RT-qPCR. There is also correspondence in the first stages of germination with a decrease expression, even though more perceptibly in the *Gln1;5*.

By the data withdrew is possible to state that both genes, *Gln1;3* and *Gln1;5*, have an undeniable role in the seed genesis, but the lack of significant results in seed yield of these single mutants (**3.3.3**) with normal seed phenotypes (**3.3.4**), and lower number of

abnormal phenotypes, leads to the conclusion that GS1 isoenzymes act in a functional redundant way during sed development in *A. thaliana*.

3.4.2.2. *gln1;3* and *gln1;5* mutants' study

After the previously described wild-type expression analysis, the torpedo, green, mature seeds and the 24 hours after germination were defined as the four most interesting predefined stages to be analyzed by RT-qPCR in *gln1;3* (SALK148604) and *gln1;5* (SALK_086579*)* mutant plants, by studying the expression of the different GS1 isogenes. At an early torpedo embryo stage both mutants have a downregulation of *Gln1;1*, the *gln1;3* mutant shows an upregulation of the *Gln1;5* gene (**Figure 38**), the *gln1;5* mutant showed a lower expression of the *Gln1;3*, and a higher expression of *Gln1;2* although not statically significant.

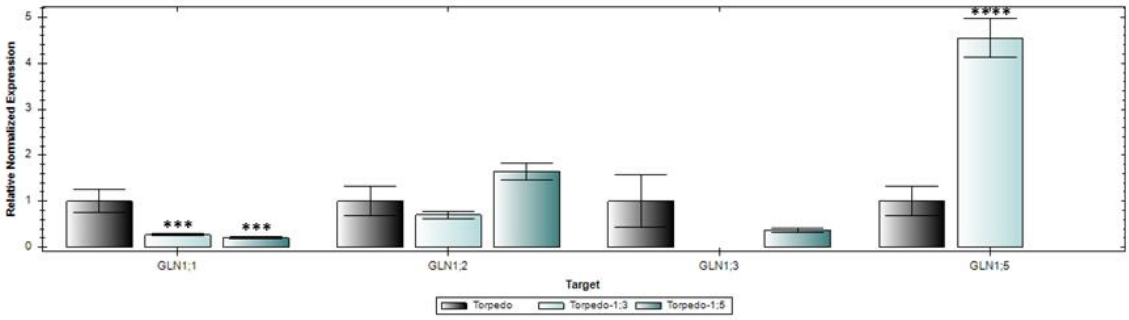


Figure 38 – Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana gln1;3* (SALK_148604), *gln1;5* (SALK_086579) mutants and wild-type seeds with the embryo in an early torpedo stage. Each bar represents the average, +/- SD (n = 3). * indicates significant differences between the *gln* mutants and wild-type plants, P ≤ 0.05. *** P ≤ 0.001. **** P ≤ 0.0001.

In green seeds (**Figure 39**), the *Gln1;1* and *Gln1;5* are upregulated for the *gln1;3* mutant. The *gln1;5* mutant had no significant differences detected in the expression levels of the other isogenes however, as for the torpedo embryos, the *Gln1;2* has higher levels of expression than wild-type plants.

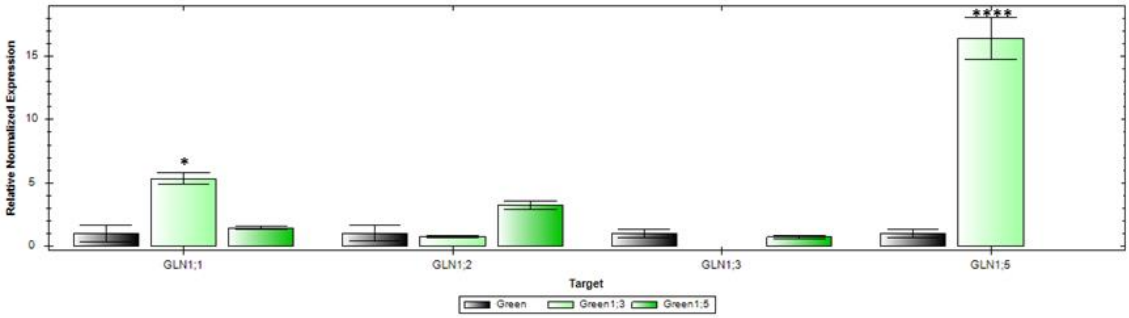


Figure 39 – Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana gln1;3* (SALK_148604) and *gln1;5* (SALK_086579) mutants compared with wild-type seeds with the embryo in a late torpedo stage (green seeds). Each bar represents the average, +/- SD (n = 3). * indicates significant differences between the *gln* mutants and wild-type plants, P ≤ 0.05. **** P ≤ 0.0001.

In mature seeds (**Figure 40**), like the green seeds, both mutants show higher *Gln1;1* expression than wild-type, but the differences are not significant. The *gln1;5* mutant turned out to have a higher (but not significant) expression of *Gln1;3*, and the *gln1;3* mutant shows again the *Gln1;5* gene upregulated.

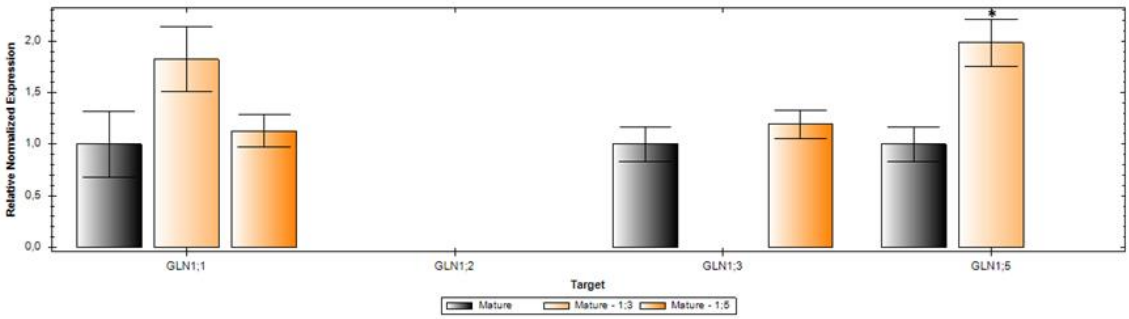


Figure 40 – Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana gln1;3* (SALK_148604) and *gln1;5* (SALK_086579) mutants compared with wild-type mature seeds. Each bar represents the average, +/- SD (n = 3). * indicates significant differences between the *gln* mutants and wild-type plants (P ≤ 0.05).

Finally, at 24 hours after germination (**Figure 41**) all genes are expressed as the wild-type ones, except *Gln1;2*, which has higher levels in both mutants, but only significant for *gln1;5* seeds.

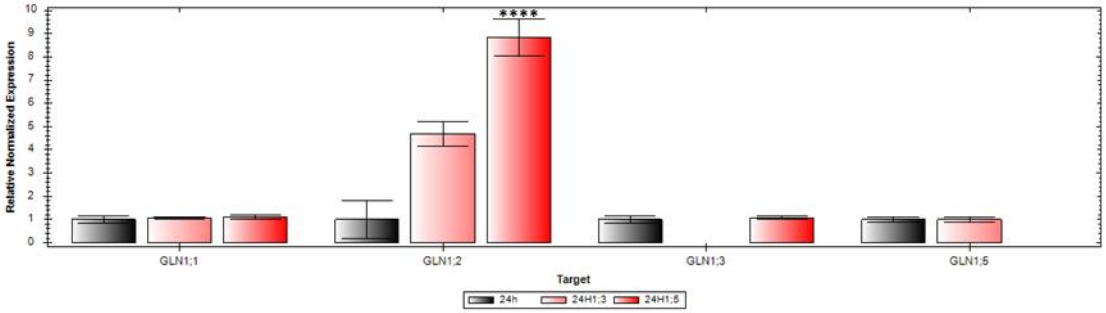


Figure 41 – Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana gln1;3* (SALK_148604) and *gln1;5* (SALK_086579) mutants compared with wild-type seeds with 24 hours germination. Each bar represents the average, +/- SD (n = 3). **** indicates significant differences between the gln mutants and wild-type plants ($P \leq 0.0001$).

These results show that *gln1;3* mutant plants have the *Gln1;1* in early torpedo seeds downregulated (**Figure 38**), but overexpressed at other seed maturation stages. The *Gln1;2* values didn't have differences during seed development, although 24 hours after the germination (**Figure 41**) the expression values are higher than wild-type, but not statistically significant. An upregulation of the *Gln1;5* gene through seed formation process occurred in *gln1;3* plants, but 24 hours after germination this overexpression was not verified.

The *gln1;5* mutant plants have (as the *gln1;3*) the *Gln1;1* downregulated in early torpedo seeds (**Figure 38**), and similar values to the wild-type plants in other seed development stages, as in 24 hours after germination. The *Gln1;2,* when expressed, had always higher values than wild-type seeds, but only 24 hours after the germination these differences are statistically significant (**Figure 41)**, being upregulated. The *Gln1;3* expression was sensibly equal to wild-type in the *gln1;5* mutant.

The data acquired suggest that the absence of *Gln1;3* can probably be primarily compensated by *Gln1;5* and *Gln1;1* during seed formation. Thus, the known *Gln1;1* compensation in *gln1;3* plants (Dragićević *et al*., 2014) occurs at seed level too. In the germination process, the *Gln1;3* is probably less essential having no statistically significant compensations, although *Gln1;2* expression was higher than wild-type seeds (**Figure 41**). This result is consistent with the *gln1;3* mutants growth assays in roots (**Figure14**), strengthen the idea that the shorter roots (**Figure 14A, 14D**) of *gln1;3* mutants in the 5th day of assay can be due to the role of GLN1;3 isoenzyme in N remobilization to roots when external N is not available, and the *gln1;3* longer shoots (**Figure 16A, 16D**), at 5 mM and 20 mM of nitrate perhaps due to a higher expression of the *Gln1;2* (**Figure 41**). The *Gln1;5* gene probably has an essential role 24 hours after germination, being highly compensated by *Gln1;2,* that can be upregulated to relieve ammonium toxicity as previously shown (Ishiyama *et al.*, 2004; Guan *et al.,* 2016). In

mature seeds stage we can see that *Gln1;2* vestigial expression, as previously shown (Schmid *et al.*, 2005 and Dragićević *et al.*, 2014), possibly reflects in a slightly higher expression of *Gln1;1* and *Gln1;3* when compared to wild-type, corroborating a possible compensation role of *Gln1;2* in *gln1;5* plants. The lower number of germinated seeds in *gln1;5* mutants (**Figure 13C**), especially at 0 mM N concentration (besides non-significant), can be a consequence of the low expression of *Gln1;2*, reinforcing its possible importance in covering the *Gln1;5*.

The downregulation of the *Gln1;1* gene in the early torpedo stage, verified in both mutants, can be related to the need of *Gln1;3* and *Gln1;5* expression to *Gln1;1* gene proper function. This can happen through a mechanism already described in soybean (Ortega *et al.*, 2012), where the 5'-UTR of one GS gene acts as a translational enhancer in specific situations. Finally, is also important to highlight the *Gln1;1* regulation mechanism, that is sensible to environmental and developmental signs (Ishiyama *et al*., 2004) leading to a specific down-regulation in some situations.

Further expression studies would be pertinent as complementary data, studying the expression levels of *Gln2*, *GDH1* and *ASN1* (GS2, glutamate dehyodrenase and asparagine synthetase, respectively) genes, to unveil some other compensation mechanisms acting in the seeds of these single mutants, as compensatory mechanisms are now believe to be of frequent occurrence in *A. thaliana* (Ferreira *et al.*, 2019). The RT-qPCR results strengthen the idea of GS genes redundancy in *Arabidopsis* (Dragićević *et al*., 2014; Guan *et al*., 2015; Moison *et al.*, 2018; Ferreira *et al.*, 2019; Ji *et al.*, 2019) as opposed to monocot species like rice (Tabuchi *et al*., 2005; Funayama *et al*., 2013) or maize (Martin *et al*., 2006). It is also mandatory the study of a possible double mutant *gln1;3*/*gln1;5*. Until now the difficulty to get this double mutant highlights the importance of these two genes in seed maturation and germination processes, deducted by the acquired data.

4. Conclusion

The central purpose of this dissertation is to contribute for a better comprehension about the role of the GS1 in the *A. thaliana* seed development and germination. The work focused on the phenotypic characterization of *Gln1;3* and *Gln1;5* null mutants, making some incursions in the earlier stages of plant sexual reproduction, the seed development and germination as well. The *gln1;3* and *gln1;5* single mutants of this work were already used in other scientific works and the results of this research validate the already known viability of both single mutants.

The radicles of *gln1;3* mutants, in the first five days of growth, were shorter than wild-types plants in the medium without nitrogen. The results suggest a determinant role for the *Gln1;3* gene in internal nitrogen remobilization to the radicle during the first days of germination. Ten and fifteen days after germination, in nitrogen excess, the *gln1;3* mutants had longer radicles than wild-type plants. The results corroborate the already known compensation mechanism by other *Gln* genes, and their possible up-regulation in N excess. By 15th day the *gln1;3* plants end up with shorter shoots when supplied with external nitrogen, being their fresh weight also lower when compared with Wt. So, the *Gln1;3* can have an influential role in the shoots when nitrogen remobilization from the exterior, or N detoxification, is needed. The *gln1;3* floral stems were fewer than the wild-type, and the *gln1;3* siliquae slightly shorter, having overall minor phenotypic changes. During seed formation the *Gln1;3* expression increases and its activity shows up dispersed through the seed coat, appearing exclusively in the embryo radicles of mature seeds. In the first 72 hours after germination its expression decreases, at 24 hours is localized at embryo's radicle and then, with diminished expression values, localized in seedlings' hypocotyl. The *Gln1;3* lack in single mutant plants causes an upregulation of the *Gln1;5* and *Gln1;1*, being the first one the highly expressed, contradicting the general idea of *Gln1;5* as an obsolete gene in the GS mechanism.

The *gln1;5* mutants in the first 15 days of growth have less mass than wild-type plants for all N concentrations, and full grown-up plants have a slightly higher number of leaves in the rosette and longer stems. Besides the plantlets lower weight and minor phenotypic changes in the grown-up plants, the *Gln1;5* gene seems to not interfere with root and shoot normal growth at the first 15 days. During seed maturation the *Gln1;5* gene expression levels increase, reaching the higher expression values in mature seeds, the most expressed GS gene. Its expected activity, marked with GUS, during seed development appears only at green seed stage with later torpedo embryos, expressing

in the embryo's cotyledons and overlapping seed tissues. From the mature seeds stage on, the *Gln1;5* expresses in all embryo and peripheral seed tissues. In the first 24 hours after germination the expression upholds higher levels in all seed tissues. Seventy-two hours after germination the expression levels have an abrupt decrease and the activity is restricted to the plantlet's hypocotyl zone. The *gln1;5* mutant plants during the seed development had the *Gln1;2* slightly higher levels of expression, ended up being upregulated by the 24 hours after germination, suggesting a relevant role, played by *Gln1;5,* in the first hours of the germination process.

Both genes have expression in flowers, being confirmed by this work that GLN1;5 is a pollen specific GS and GLN1;3 an isoenzyme with a much wider activity, expressing also in the female floral organs, with higher intensity in the flowers' stigma. The lack of these genes in the single mutants did not turn to be determinant to the pollen integrity and viability or to compromise the fertilization process. Thus, the pertinence to study the *gln1;3*/*gln1;5* double mutant is higher, nonetheless the difficulty to find it and the germination assay results suggest that the lack of these two genes can compromise the seed integrity and/or the germination process.

The door stays open to further research on the GS role in seed development and germination. The study of *Gln1;3* and *Gln1;5* genes, in the terms of this research, sheds light to an important and, so far, underexplored topic with many unanswered questions, still waiting for future prospects.

5. Future perspectives

This research allowed the confirmation that both target genes (*Gln1;3* and *Gln1;5*) are highly expressed in *A. thaliana* seeds, concluding that their individual lack promoted some minor alterations in the plants' phenotype. It was shown in this work that GS1 isoenzymes have the capacity to counterbalance the lack of each one of the isoenzymes studied, promoting this compensation mechanism a robustness in the *A. thaliana* organism. So, is pertinent the study of double or even high order mutants, to a more detailed characterization of this gene family in seed's development and germination. The predicted interaction of *Gln1;1* gene with *Gln1;5* (between other pertinent interacting genes) turns the so far unproduced double mutant gln1;1/gln1;5 also a relevant target. More research is also needed to confirm the *gln1;3*/*gln1;5* unviability or, if viable, its possible study and characterization. Expression studies in related enzymes as glutamate dehyodrenase and asparagine synthetase are also approaches of interest, appropriate to help in the GS mutants' characterization. All the knowledge will be important not only for a better comprehension about the GS role in seed development, but also to promote the future production of seeds with higher yields of production, higher N efficiency use and reducing the necessity of external nitrogen provision, leading to higher productivity with less crop area, lower environmental contaminations and less impact in the global warming.

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Yang J, Sardar HS, Mcgovern KR, Zhang Y, Showalter AM, Program CB (2007) A lysine-rich arabinogalactan protein in *Arabidopsis* is essential for plant growth and development , including cell division and expansion. Plant J 629–640

7. Supplements

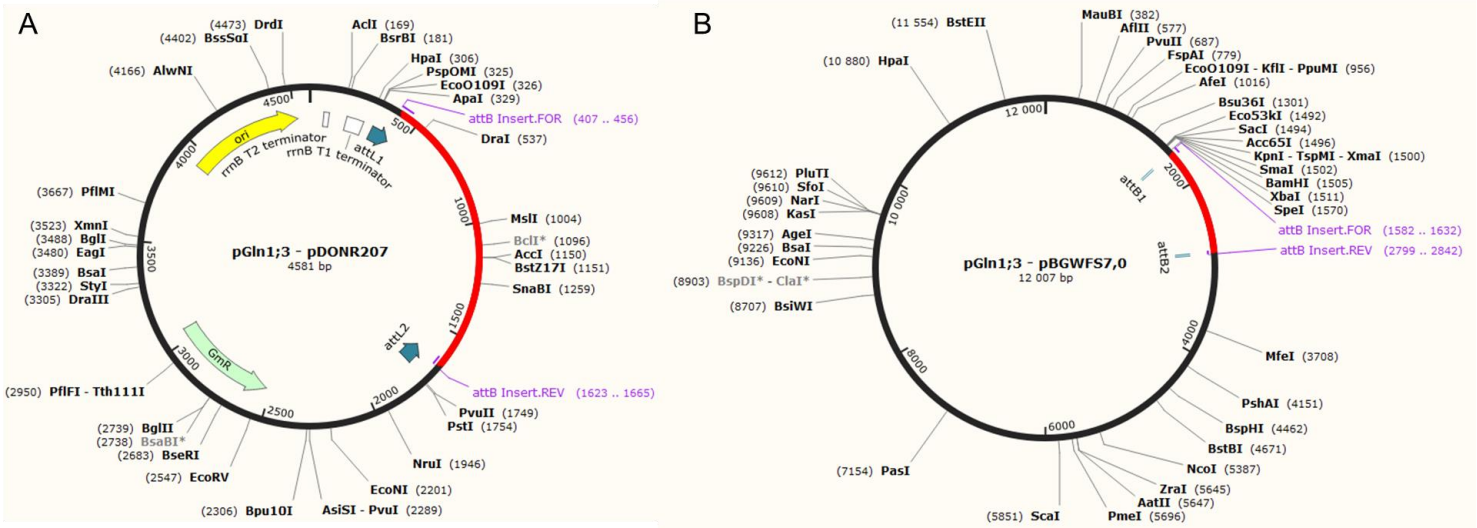

Figure S1 – Schematic representation of the pDONR™207 (Invitrogen™) entry vector (**A**) and the pBGWFS7,0 (Invitrogen™) destination vector (**B**) with the *Gln1;3* promoter.

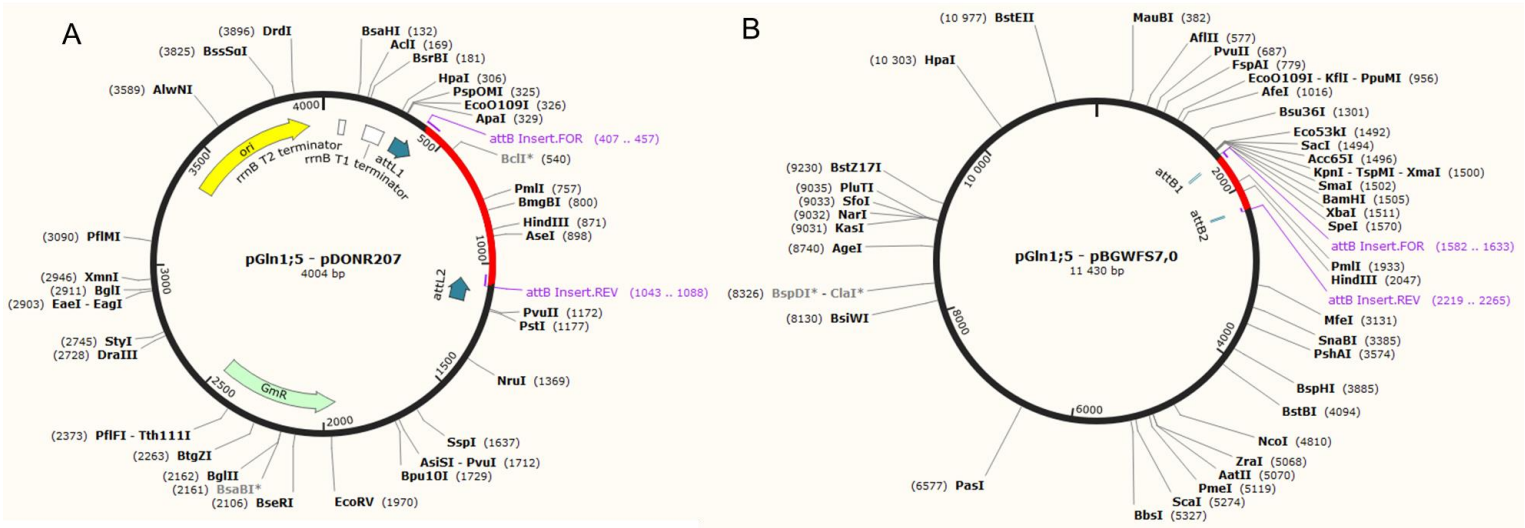

Figure S2 – Schematic representation of the pDONR™207 (Invitrogen™) entry vector (**A**) and the pBGWFS7,0 (Invitrogen™) destination vector (**B**) with the *Gln1;5* promoter.

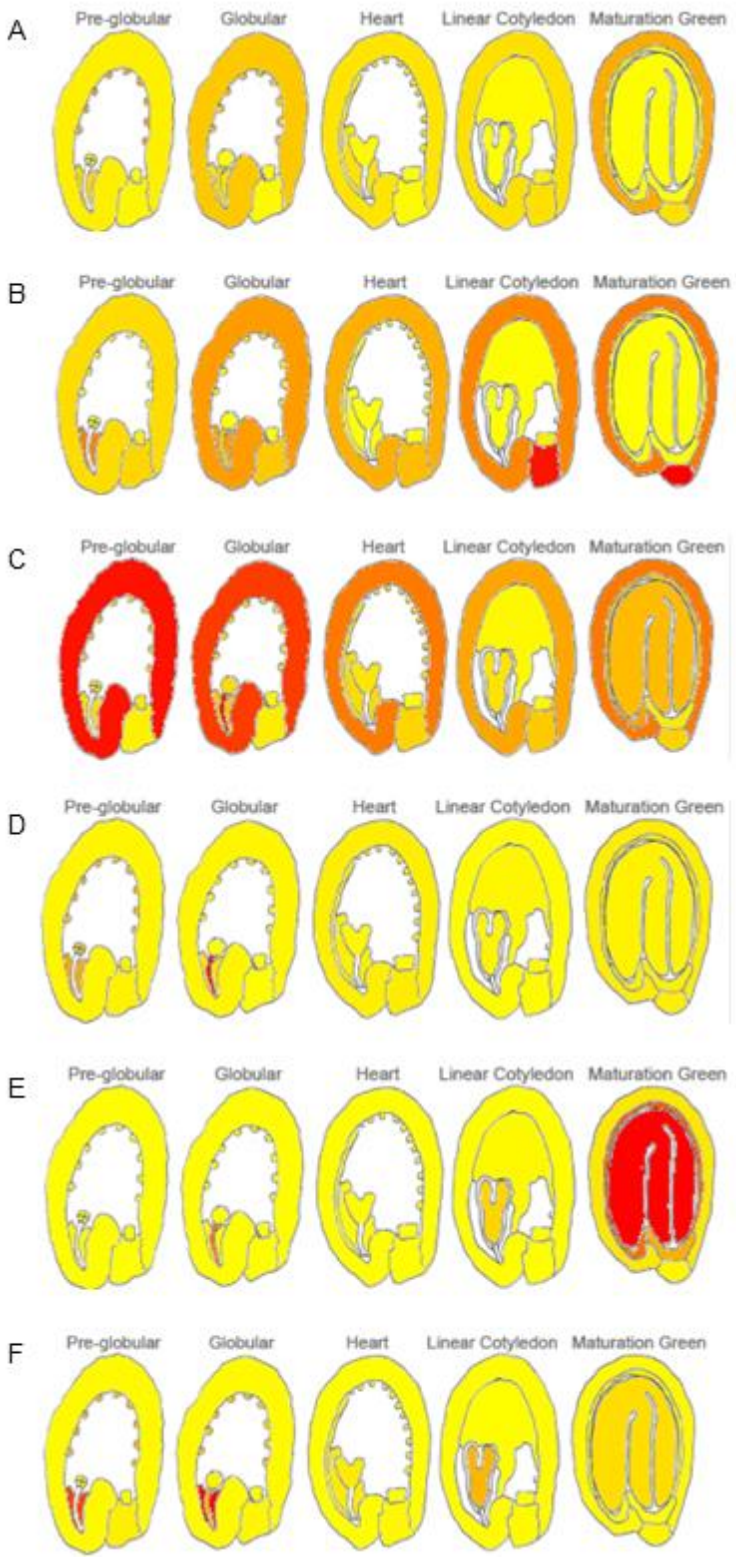

Figure S3 – GS genes expression patterns in seed tissues during the seed maturation. **A –** *Gln1;1*; **B –** *Gln1;2*; **C –** *Gln1;3*; **D –** *Gln1;4*; **E –** *Gln1;5*; **F –** *Gln2*.

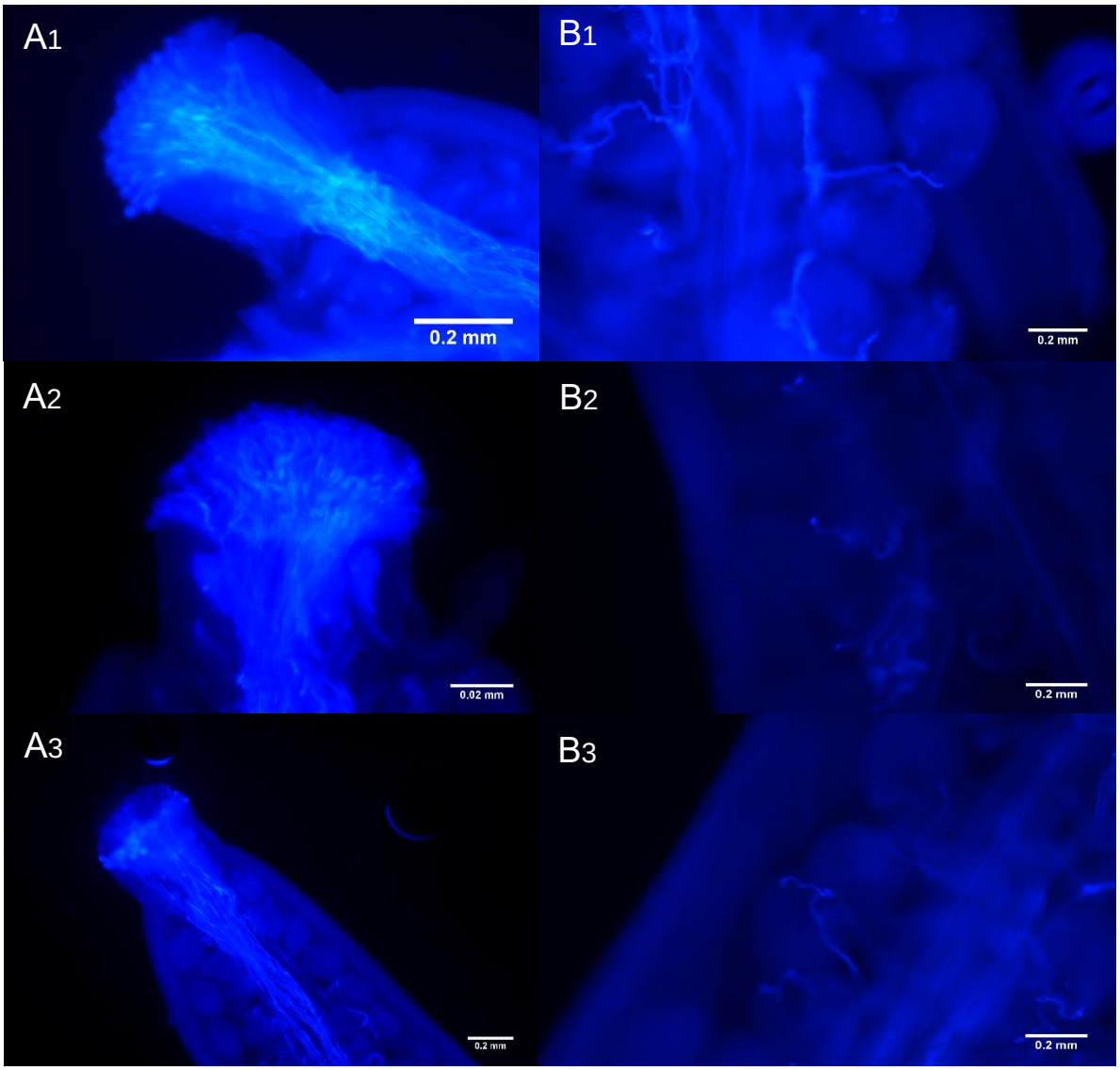

Figure S4 – Aniline blue staining after reciprocal crosses between Wt ♀ x Wt ♂ (**A1**, **B1**), *gln1;3* ♂ x *gln1;3* ♀ (**A2**, **B2**) and *gln1;5* ♂ x *gln1;5* ♀ (**A3**, **B3**). **A1**, **A2**, **A3 –** Pollen grains germinating in the flower's pistil. **B1**, **B2**, **B3 –** Pollinic tubes growing into the embryo sac.

A

```
promoter1.3       ------------------------------------------------------------
        0
pDORN             aaggattttattttgactgatagtgacctgttcgttgcaacaaattgatgagcaatgctt
        60


promoter1.3       ----------------------------------gttagtagcctgatgaagttatgac
        25
pDORN             ttttataatgccaactttgtacaaaaaagcaggctgttagtagcctgatgaagttatgac
        120
                                                   *************************

promoter1.3       cacccaaatatttcaataaaggttcagcaacttcattcacaactcacatgcaaattacga
        85
pDORN             cacccaaatatttcaataaaggttcagcaacttcattcacaactcacatgcaaattacga
        180
                  ************************************************************

promoter1.3       aggctctttctttcaaatgtttaaaatcaacttcttaaatctcacttgaaaagaaataac
        145
pDORN             aggctctttctttcaaatgtttaaaatcaacttcttaaatctcacttgaaaagaaataac
        240
                  ************************************************************

promoter1.3       taaattttatgtttcttaattttcttctcgttgtgtattattcatcttgtatccatccat
        205
pDORN             taaattttatgtttcttaattttcttctcgttgtgtattattcatcttgtatccatccat
        300
                  ************************************************************

promoter1.3       agcaaccatttctttgttacttcaagttggcttaaattttggccaataataataccatag
        265
pDORN             agcaaccatttctttgttacttcaagttggcttaaattttggccaataataataccatag
        360
                  ************************************************************

promoter1.3       accttctccatgaagcaagatgttgcggtagtaacctttttattaaggtgtgatttggcta
        325
pDORN             accttctccatgaagcaagatgttgcggtagtaacctttttattaaggtgtgatttggcta
        420
                  ************************************************************

promoter1.3       aagaaacttggtcttatatcattaaaattagatattgaacaaaatcaagtatgagctgat
        385
pDORN             aagaaacttggtcttatatcattaaaattagatattgaacaaaatcaagtatgagctgat
        480
                  ************************************************************

promoter1.3       agaaccaagattagtaggaagctaatcaaacaactctggatacatttactaggaaaatat
        445
pDORN             agaaccaagattagtaggaagctaatcaaacaactctggatacatttactaggaaaatat
        540
                  ************************************************************

promoter1.3       ggtcatgcttatttttatacgcatcatttgaatccgaagaggggaaaaaggcaacaaaac
        505
pDORN             ggtcatgcttatttttatacgcatcatttgaatccgaagaggggaaaaaggcaacaaaac
        600
                  ************************************************************

promoter1.3       gggcttaatgagaaattgactatggaagaaatatcaaaaagccaaaagtggccacgactt
        565
```

```
pDORN             gggcttaatgagaaattgactatggaagaaatatcaaaaagccaaaagtggccacgactt
        660
                  ************************************************************

promoter1.3       tctgcatcgcaatgatacctatattagaagctacttaaaaattttaacacaaaaaagaaa
        625
pDORN             tctgcatcgcaatgatacctatattagaagctacttaaaaattttaacacaaaaaagaaa
        720
                  ************************************************************

promoter1.3       aacatttagtttcctcctaccttacctacctacgtttacgtgatcatcaacaattctatt
        685
pDORN             aacatttagtttcctcctaccttacctacctacgtttacgtgatcatcaacaattctatt
        780
                  ************************************************************

promoter1.3       tttactaatttactcattaatatatcttctaaagtatacataaacaaacaaactgtacga
        745
pDORN             tttactaatttactcattaatatatcttctaaagtatacataaacaaacaaactgtacga
        840
                  ************************************************************

promoter1.3       agaaattgaaatataaatatcaaggaaattggtagcagttgttgagttatttcaatgatg
        805
pDORN             agaaattgaaatataaatatcaaggaaattggtagcagttgttgagttatttcaatgatg
        900
                  ************************************************************

promoter1.3       ctgtgaagtggaaacatttattacgtaacgcatatccgttttgtatataaactcttattt
        865
pDORN             ctgtgaagtggaaacatttattacgtaacgcatatccgttttgtatataaactcttattt
        960
                  ************************************************************

promoter1.3       ttcggctgtatatataatctttttagagattttccgtgagaaatagattaaatattgttg
        925
pDORN             ttcggctgtatatataatctttttagagattttccgtgagaaatagattaaatattgttg
        1020
                  ************************************************************

promoter1.3       ccataataacctgtaatctcaggagaatcctaacggaaaagagaatattagacgatagta
        985
pDORN             ccataataacctgtaatctcaggagaatcctaacggaaaagagaatattagacgatagta
        1080
                  ************************************************************

promoter1.3       ttatatggagtattaattacttattaacaattctaggagataatatcagcacacaacaat
        1045
pDORN             ttatatggagtattaattacttattaacaattctaggagataatatcagcccccac-aat
        1139
                  ************************************************** * **  ***

promoter1.3       aaaagaaattactaatttactggaatcatgtattcaccgtaattatttaaggagaggaaa
        1105
pDORN             aaaagaaattactaatttactggaatcatgtattccccgtattatttagggaaaggaaaa
        1199
                  ********************************** ***** *  **   * *  * ***

promoter1.3       aaaaaaatacaagctctttctctataaacacacactctcaggagagaagttgtattgatc
        1165
pDORN             aaaa---tacaagctctttcctata---aacccacttctcaggaaaaaattggattgatc
        1253
                  ****   *************         ** ****     *  * ** *** *******

promoter1.3       gtcttctctttccctaaacacactgattattttctctccgacgccgcc------------
        1213
```

```
pDORN              gccttcctttccctaaaccc---tgattattttctcccacccccacccccttttcttgtaa
        1310
                 * ****  ** **  ** *    ************ *   * *  **

promoter1.3        ------------------------------------------------------------
        1213
pDORN              aaattggctttaaaaaaaacttggctaaaatttgtgcacgaaagggccttatccccaaaa
        1370


promoter1.3        ------------------------------------------------------------
        1213
pDORN              aaatttttttttgccccccgctgcccctgggcgggtccaaacttgggttattgcaaaaaaaa
        1430


promoter1.3        ------------------------------------------------------------
        1213
pDORN              attcctgggaaaaaaggttcgtttcaaaaataacgggggttggcattcgggcgcggcccc
        1490


promoter1.3       ---------------------------        1213
pDORN             ttttttccccgggtttttgaagggccg        1516
```

B

```
1.3revcomp       ------------------------------------------------------------ 0
pDONR            aatggattttattttgactgatagtgacctgttcgttgcaacatattgataagcaatgct 60


1.3revcomp       ----------------------------------ggcggcgtcggagagaaaataatcag 26
pDONR            ttcttataatgccaactttgtacaagaaagctgggtcggcgtcggagagaaaataatcag 120
                                                  * ************************


1.3revcomp       tgtgtttagggaaagagaagacgatcaatacaacttctctcctgagagtgtgtgtttata 86
pDONR            tgtgtttagggaaagagaagacgatcaatacaacttctctcctgagagtgtgtgtttata 180
                 ************************************************************


1.3revcomp       gagaaagagcttgtatttttttttttcctctccttaaataattacggtgaatacatgattc 146
pDONR            gagaaagagcttgtattttt-ttttcctctccttaaataattacggtgaatacatgattc 239
                 ******************* ****************************************


1.3revcomp       cagtaaattagtaatttcttttattgttgtgtgctgatattatctcctagaattgttaat 206
pDONR            cagtaaattagtaatttcttttattgttgtgtgctgatattatctcctagaattgttaat 299
                 ************************************************************


1.3revcomp       aagtaattaatactccatataatactatcgtctaatattctcttttccgttaggattctc 266
pDONR            aagtaattaatactccatataatactatcgtctaatattctcttttccgttaggattctc 359
                 ************************************************************


1.3revcomp       ctgagattacaggttattatggcaacaatatttaatctatttctcacggaaaatctctaa 326
pDONR            ctgagattacaggttattatggcaacaatatttaatctatttctcacggaaaatctctaa 419
                 ************************************************************


1.3revcomp       aaagattatatatacagccgaaaaataagagtttatatacaaaacggatatgcgttacgt 386
pDONR            aaagattatatatacagccgaaaaataagagtttatatacaaaacggatatgcgttacgt 479
                 ************************************************************


1.3revcomp       aataaatgtttccacttcacagcatcattgaaataactcaacaactgctaccaatttcct 446
pDONR            aataaatgtttccacttcacagcatcattgaaataactcaacaactgctaccaatttcct 539
                 ************************************************************


1.3revcomp       tgatatttatatttcaatttcttcgtacagtttgtttgtttatgtatactttagaagata 506
pDONR            tgatatttatatttcaatttcttcgtacagtttgtttgtttatgtatactttagaagata 599
                 ************************************************************
```

```
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pDONR           tattaatgagtaaattagtaaaaatagaattgttgatgatcacgtaaacgtaggtaggta 659
                ************************************************************

1.3revcomp      aggtaggaggaaactaaatgtttttctttttgtgttaaaatttttaagtagcttctaat 626
pDONR           aggtaggaggaaactaaatgtttttctttttgtgttaaaatttttaagtagcttctaat 719
                ************************************************************

1.3revcomp      ataggtatcattgcgatgcagaaagtcgtggccacttttggctttttgatatttcttcca 686
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                ************************************************************

1.3revcomp      tagtcaatttctcattaagcccgttttgttgccttttttcccctcttcggattcaaatgat 746
pDONR           tagtcaatttctcattaagcccgttttgttgccttttttcccctcttcggattcaaatgat 839
                ************************************************************

1.3revcomp      gcgtataaaaataagcatgaccatattttcctagtaaatgtatccagagttgtttgatta 806
pDONR           gcgtataaaaataagcatgaccatattttcctagtaaatgtatccagagttgtttgatta 899
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1.3revcomp      gcttcctactaatcttggttctatcagctcatacttgattttgttcaatatctaatttta 866
pDONR           gcttcctactaatcttggttctatcagctcatacttgattttgttcaatatctaatttta 959
                ************************************************************

1.3revcomp      atgatataagaccaagtttctttagccaaatcacaccttaataaaaggttactaccgcaa 926
pDONR           atgatataagaccaagtttctttagcca-atcacaccttaataaaaggttactaccgcaa 1018
                **************************** ***************************

1.3revcomp      catcttgcttcatggagaaggtctatggtattattattggccaaaatttaagccaacttg 986
pDONR           catcttgcttcatggagaagg-ctatggtattattattggccaaaatttaagccaacttg 1077
                ********************* ************************************

1.3revcomp      aagtaacaaagaaatggttgctatggatggatacaagatgaataatacacaacgagaaga 1046
pDONR           aagtaacaaaaaatg-gttgctatggatggatccagatgataattcccaccgggaaa--- 1133
                ********** **   ********** **      *     ***   ** *

1.3revcomp      aaattaagaaacataaaatttagttatttcttttcaagtgagatttaagaagttgatttt 1106
pDONR           -aaattagaaacataaaat-tagttatttctttc----aggggaattaaaagttgatttt 1187
                 ** * ************ ************      * *   * * ***********

1.3revcomp      aaacatttgaaagaaagagccttcgtaatttgcatgtgagttgtgaatgaagttgctgaa 1166
pDONR           aacattggaaaaaa--aagcctgc------------------------------------ 1209
                **   *   *** *   ***** *

1.3revcomp      cctttattgaaatatttgggtggtcataacttcatcaggctactaac     1213
pDONR           ------------------------------------------------     1209
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C

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promoter1.5       ------------------------------------------------------------
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pDORN1.5          ggaaatgattttattttgactgatagtgacctgttcgttgcaacaaattgatgagcaatg
        60


promoter1.5       ---------------------------------------atctcagtgtttagtgattgtt
        22
pDORN1.5          ctttttttataatgccaactttgtacaaaaaagcaggctatctcagtgtttagtgattgtt
        120
                                                          **********************

promoter1.5       agatgtagccaaatcccatcggttttgttttgtttctgtgttcatttcagtagtaatgaa
        82
```

```
pDORN1.5          agatgtagccaaatcccatcggttttgttttgtttctgtgttcatttcagtagtaatgaa
        180
                  ************************************************************

promoter1.5       ttgtattaagtcactttaagaattggttgatcatgtgaaatgagaattggctggaaatgt
        142
pDORN1.5          ttgtattaagtcactttaagaattggttgatcatgtgaaatgagaattggctggaaatgt
        240
                  ************************************************************

promoter1.5       tatagaacgatcctacttaacttcagtatttgatgagatttcttattggagactcttgtg
        202
pDORN1.5          tatagaacgatcctacttaacttcagtatttgatgagatttcttattggagactcttgtg
        300
                  ************************************************************

promoter1.5       acccctttctctaaagtttcaatgtgtgaaatcacaacataagtaacacaacaatagatgg
        262
pDORN1.5          acccctttctctaaagtttcaatgtgtgaaatcacaacataagtaacacaacaatagatgg
        360
                  ************************************************************

promoter1.5       gtctggtctcagcttgtgttttggtcctttgtatgcaacaatagagaagctgaatgctta
        322
pDORN1.5          gtctggtctcagcttgtgttttggtcctttgtatgcaacaatagagaagctgaatgctta
        420
                  ************************************************************

promoter1.5       tccacgtggaagatttgtgcctccgataggtgtctttaacagtgtgacgtgtctgtattg
        382
pDORN1.5          tccacgtggaagatttgtgcctccgataggtgtctttaacagtgtgacgtgtctgtattg
        480
                  ************************************************************

promoter1.5       tttcattgatgaatctgttacaccgttgagtgattgattctcaaataaattggtcccaaa
        442
pDORN1.5          tttcattgatgaatctgttacaccgttgagtgattgattctcaaataaattggtcccaaa
        540
                  ************************************************************

promoter1.5       gctttttatcaatatgtgatggttattaatggatacttaataaaagatgccattatacta
        502
pDORN1.5          gctttttatcaatatgtgatggttattaatggatacttaataaaagatgccattatacta
        600
                  ************************************************************

promoter1.5       atcaacgattattgttgagaaaacaataaaataggatctcaagagaaatagccgcttcta
        562
pDORN1.5          atcaacgattattgttgagaaaacaataaaataggatctcaagagaaatagccgcttcta
        660
                  ************************************************************

promoter1.5       taaaaatagtgagagtgactgagatcaatagaaccaatctcagaatcatcttctttctct
        622
pDORN1.5          taaaaatagtgagagtgactgagatcaatagaaccaatctcagaatcatcttctttctct
        720
                  ************************************************************

promoter1.5       ttcggaacaaaa----------------------------------------------
        634
pDORN1.5          ttcggaacaaaaacccagctttcttgtacaaagttggcattataagaaagcattgcttat
        780
                  ************

promoter1.5       ------------------------------------------------------------
        634
```

```
pDORN1.5          caatttgttgcaacgaacaggtcactatcagtcaaaataaaatcattatttgccatccgg
        840

promoter1.5       ------------------------------------------------------------
        634
pDORN1.5          ctgcagctctggcccgtgtctcaaaatctctgatgttacattgcacaagataaaatatat
        900

promoter1.5       ------------------------------------------------------------
        634
pDORN1.5          catcatgaacaataaaactgtctgcttacataaacagtaatacaaggggtgttatgagcc
        960

promoter1.5       ------------------------------------------------------------
        634
pDORN1.5          atattcaacgggaaacgtcgaggccgcgattaaattccaacatggatgctgatttatatg
        1020

promoter1.5       ------------------------------------------------------------
        634
pDORN1.5          ggtataaatgggctcgcgataatgtcgggcaatcaggtgcgaaaatctatcgcttgtatg
        1080

promoter1.5       ------------------------------------------------------------
        634
pDORN1.5          ggaagcccgatgcgccaaagttgtttctgaaaatggcaaaggtagcgttgccaatgatgt
        1140

promoter1.5       ---------------------------       634
pDORN1.5          tacaaatgaaagggccaactaaactct       1167
```

Figure S5 – Sequencing data from the transformed pDONR207 plasmids. **A –** Plasmid transformed with *Gln1;3* promoter, forward alignment; **B –** Plasmid transformed with *Gln1;3* promoter, reverse alignment; **C –** Plasmid transformed with *Gln1;5* promoter, forward alignment;

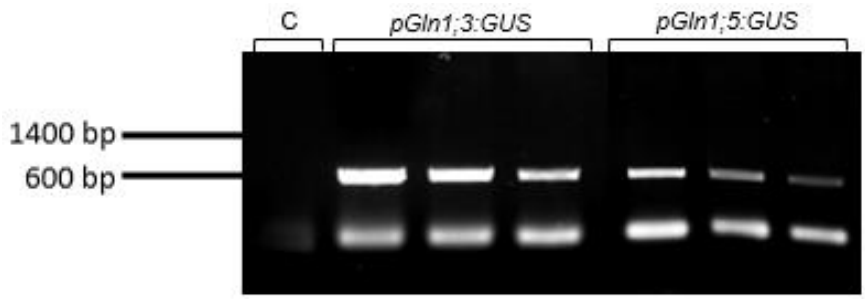

Figure S6 – Electrophoresis corresponding to the transgenic mutant lines genotyping results. **C –** Wild-type gDNA, negative control; ***pGln1;3:GUS* –** transgenic mutant plants with the *Gln1;3* promoter in the construct; ***pGln1;5:GUS* –** transgenic mutant plants with the *Gln1;5* promoter in the construct; **M.M. –** Molecular size marker.

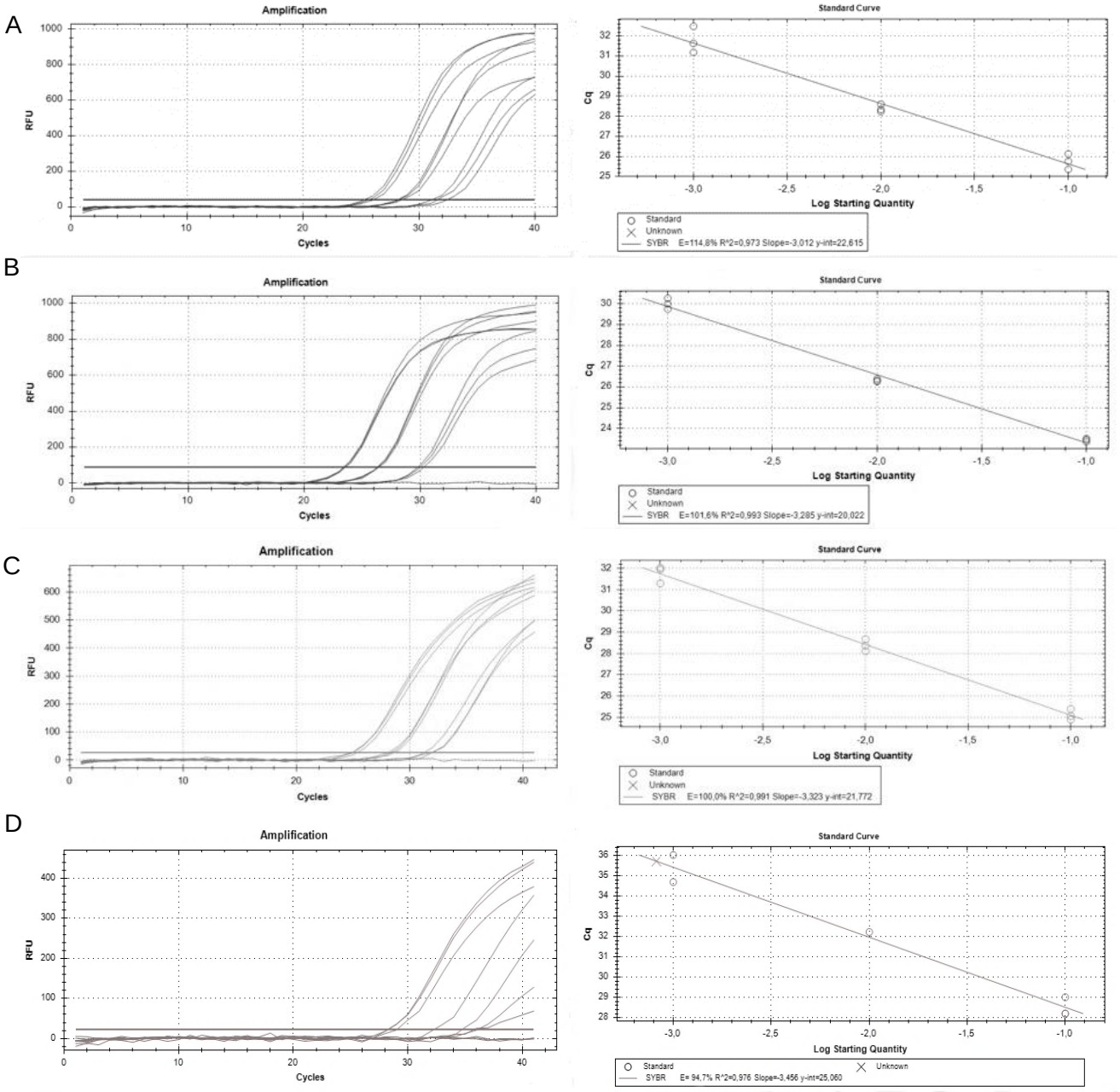

Figure S7 – Efficiency test results of the selected primers (**Table 4**) for RT-qPCR use. **A –** *Gln1;1* (114,8% of efficiency); **B –** *Gln1;2* (101,6% of efficiency); **C –** *Gln1;3* (100% of efficiency); **D –** *Gln1;5* (94,7% of efficiency).

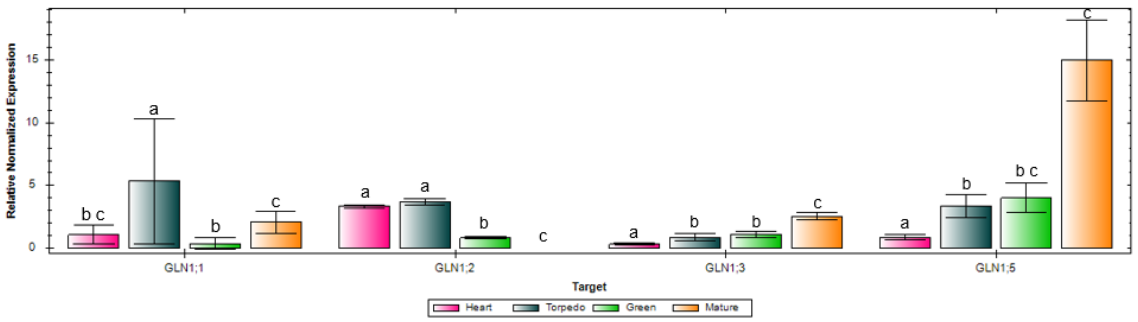

Figure S8 – Relative transcript quantity of the *Gln1;1*, *Gln1;2*, *Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana* wild-type seeds in different stages of embryo development (heart shape, torpedo, green and mature seeds). Each bar represents the average, +/- SD (n = 3). The different letters (a, b, c,) define significant differences (P ≤ 0.05) between the seed development stages for each of the *Gln* genes.

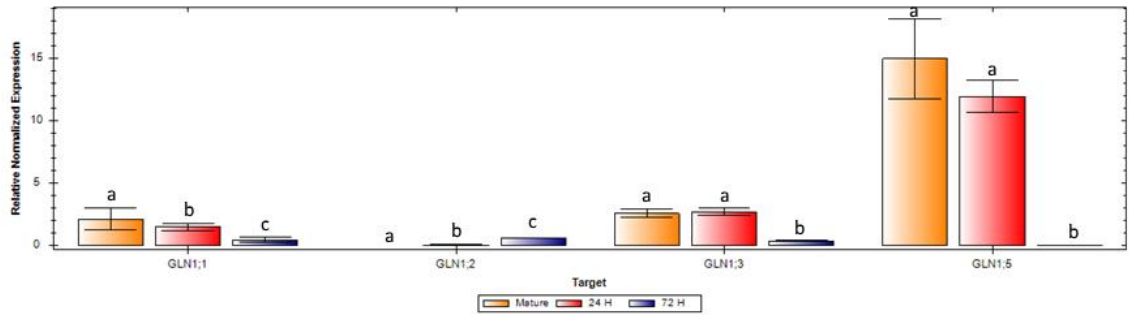

Figure S9 – Relative transcript quantity of the *Gln1;1*, *Gln1;2, Gln1;3* and *Gln1;5* genes in *Arabidopsis thaliana* wild-type seeds during the germination process (mature seeds, 24 hours and 72 hours after germination). Each bar represents the average, +/- SD (n = 3). The different letters define significant differences (P ≤ 0.05) between the seed development stages for each of the *Gln* genes.

Table S1 – Significant differences in roots and shoots growth assays expressed in P values.

	Roots									Shoots								
Assay	SALK_072283 (*gln1;3*)			SALK_148604 (*gln1;3*)			SALK_086579 (*gln1;5*)			SALK_072283 (*gln1;3*)			SALK_148604 (*gln1;3*)			SALK_086579 (*gln1;5*)		
Day	5th	10th	15th	5th	10th	15th	5th	10th	15th	5th	10th	15th	5th	10th	15th	5th	10th	15th
Wt (0 mM) vs. Wt (5 mM)	-	-	-	-	-	-	-	-	-	<0.00001	<0.00001	<0.00001	-	-	<0.00001	-	-	-
Wt (0 mM) vs. Wt (20 mM)	0.0088	-	-	-	-	0.0432	-	-	-	<0.00001	<0.00001	<0.00001	-	<0.00001	<0.00001	-	-	0.0032
Wt (5 mM) vs. Wt (20 mM)	-	0.0228	0.0142	0.0009	-	0.0390	-	-	-	-	-	-	0.0436	0.0013	-	-	-	0.0104
***gln* (0 mM) vs. *gln* (5 mM)**	-	-	-	-	-	-	-	-	-	<0.00001	<0.00001	<0.00001	-	<0.00001	<0.00001	-	-	-
***gln* (0 mM) vs. *gln* (20 mM)**	-	0.0008	-	0.0002	<0.00001	<0.00001	-	-	-	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	-	0.0213	0.0021
***gln* (5 mM) vs. *gln* (20 mM)**	-	0.0267	-	-	<0.00001	<0.00001	-	-	-	-	-	-	0.0436	0.0011	-	-	-	-
Wt (0 mM) vs. *gln* (0 mM)	0.0319	-	-	0.0006	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wt (5 mM) vs. *gln* (5 mM)	-	-	-	-	-	-	-	-	-	0.0034	0.0032	-	0.0348	-	<0.00001	-	-	-
Wt (20 mM) vs. *gln* (20 mM)	-	0.0411	0.0411	-	<0.00001	<0.00001	-	-	-	<0.00001	<0.00001	0.0438	0.0348	0.0443	<0.00001	-	-	-

Table S2 – Significant differences in fresh weight assays expressed in P values.

	Fresh weight		
Assay	SALK_072283 (*gln1;3*)	SALK_148604 (*gln1;3*)	SALK_086579 (*gln1;5*)
Wt (0 mM) vs. Wt (5 mM)	<0.0001	<0.0001	<0.0001
Wt (0 mM) vs. Wt (20 mM)	<0.0001	<0.0001	<0.0001
Wt (5 mM) vs. Wt (20 mM)	<0.0001	0.0214	-
gln (0 mM) vs. *gln* (5 mM)	<0.0001	0.0004	0.0010
gln (0 mM) vs. *gln* (20 mM)	<0.0001	<0.0001	<0.0001
gln (5 mM) vs. *gln* (20 mM)	<0.0001	0.0016	0.0053
Wt (0 mM) vs. *gln* (0 mM)	-	-	-
Wt (5 mM) vs. *gln* (5 mM)	0.0221	0.0036	0.0004
Wt (20 mM) vs. *gln* (20 mM)	0.0003	0.0495	0.0218

Table S3 – Significant differences in the morphological assays expressed in P values.

Assay	Mutant	P value
Floral stems length	*gln1;5* (SALK_086579)	0.0021
Number of floral stems	*gln1;3* (SALK_148604)	0.0445
Number of leaves	*gln1;5* (SALK_086579)	0.0033
Siliquae length	*gln1;3* (SALK_072283; SALK_148604)	0.0106; 0.0337